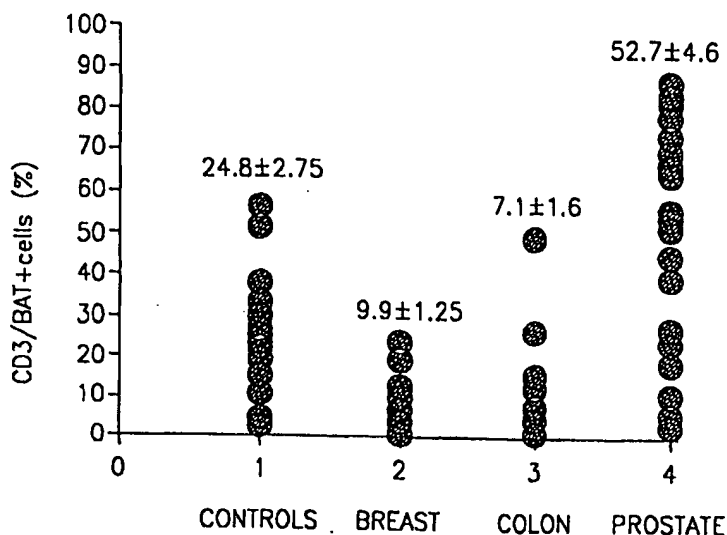


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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C07K 16/18, 16/28, 7/04, A61K 39/395, 38/04, G01N 33/574, C12N 5/12, 5/16, 15/13, 15/63		A1	(11) International Publication Number: WO 00/58363 (43) International Publication Date: 5 October 2000 (05.10.00)
(21) International Application Number: PCT/IL99/00518 (22) International Filing Date: 30 September 1999 (30.09.99) (30) Priority Data: 129299 31 March 1999 (31.03.99) IL (71) Applicant (for all designated States except US): MOR-RESEARCH APPLICATIONS LTD. [IL/IL]; 23 Hasivim Street, Kiriath Matalon, P.O. Box 7590, 49170 Petach Tikva (IL). (72) Inventors; and (75) Inventors/Applicants (for US only): HARDY, Britta [IL/IL]; 6 Eliahu Hakim Street, 69120 Tel-Aviv (IL). NOVOGRODSKY, Abraham [IL/IL]; 18 Shderot Chen Street, 76469 Rehovot (IL). (74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel-Aviv (IL).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	

(54) Title: MONOCLONAL ANTIBODIES, ANTIGENS AND DIAGNOSIS AND THERAPY OF MALIGNANT DISEASES**(57) Abstract**

The invention concerns novel DNA and amino acid sequences of monoclonal antibodies (mAbs) raised against lymphoblastoid cells and peptides to which the mAbs bind to. The invention also concerns diagnostic assays using said antibodies or peptides for detecting individuals with a high probability of having a malignant disease and, at times, for detecting an individual having a specific malignant disease. The invention further concerns pharmaceutical compositions comprising the mAbs or peptides of the invention for use in the treatment of various malignant diseases as well as methods for the treatment of malignant diseases using the mAbs or peptides of the invention.

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MONOCLONAL ANTIBODIES, ANTIGENS AND DIAGNOSIS AND THERAPY OF MALIGNANT DISEASES

FIELD OF THE INVENTION

The present invention concerns novel sequences of monoclonal antibodies, peptidic sequences of antigens to which the monoclonal antibodies bind, as well as diagnostic and therapeutic assays using the monoclonal antibody and peptides.

5

BACKGROUND OF THE INVENTION

Co-owned PCT Application, Publication No. WO 95/20605, discloses immuno-stimulatory monoclonal antibodies. The antibodies subject of this PCT application were raised against B lymphoblastoid cells and were shown to have an
10 immuno-stimulatory effect. When injected into tumor-bearing animals, these antibodies were also found to elicit an anti-tumor effect.

Cancer diagnosis, under current medical procedures, is typically a multi-step process involving physical examination, use of a variety of imaging techniques, employment of a variety of cancer markers, etc. There is a longfelt need in the art
15 for cancer diagnostic techniques which allow detection of cancer and also determination of the type of cancer which the tested individual is suffering from.

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GENERAL DESCRIPTION OF THE INVENTION

The present invention is based on the finding of sequences of monoclonal antibodies against lymphoblastoid cells. The present invention is further based on the finding that the level of binding of these antibodies to T-cells of patients having
5 cancer is different (higher or lower) than the level of binding of these antibodies to T-cells of healthy individuals.

In accordance with one aspect of the invention there is provided a monoclonal antibody having a variable region selected from the group consisting of:

- 10 (a) a monoclonal antibody having a heavy chain variable region comprising the amino acid sequence of Fig. 1;
- (b) a monoclonal antibody having a Kappa light chain variable region comprising the amino acid sequence of Fig. 2;
- (c) a monoclonal antibody having a heavy chain variable region
15 comprising the amino acid sequence of Fig. 1 and the Kappa light chain variable region comprising the amino acid sequence of Fig. 2;
- (d) a monoclonal antibody having a heavy chain variable region having at least 70% identity to the amino acid sequence of Fig. 1;
- (e) a monoclonal antibody having a light chain variable region having at
20 least 70% identity to the sequence of Fig. 2.

In accordance with the invention, the term "*antibody*" refers to monoclonal antibodies of any of the classes IgG, IgM, IgD, IgA and IgE. The term refers to whole antibodies or fragments of the antibodies comprising the antigen-binding domain of the antibodies, e.g. antibodies lacking the Fc portion, single chain
25 antibodies, fragments of articles consisting essentially of only the variable antigen-binding domain of the antibody, etc.

In addition the invention also concerns antibodies which bind to an antigen to which any one of the above mAbs specifically binds to i.e. antibodies which have cross reactivity with the above antibodies.

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In accordance with one embodiment of the invention, the monoclonal antibody is a chimeric human-mouse antibody, namely a mAb with a constant region derived from a human origin and a variable region derived from mouse. For this purpose, the Kappa light and heavy chain variable regions of the mAb of the invention were PCR cloned and their DNA sequenced. In accordance with yet another embodiment of the invention the antibody is a fully humanized antibody, i.e. both its variable and constant region are derived from a human source.

The term "*having at least X percent identity*" refers to the percent of amino acid residues that are identical in the two compared sequences when the sequences are optimally aligned. Thus, 70% amino acid sequence identity means that 70% of the amino acids in two or more optimally aligned polypeptide sequences are identical. Preferably, the identity is at least 80%, most preferably at least 90%.

In accordance with an additional aspect of the invention, there are provided mouse hybridoma cell lines which produce any of the mAbs of the invention. The hybridomas may be prepared by any of the methods known in the art (for example, Kohler, G. and Milstein, C., *Nature*, 256:495-497, (1975)). The supernatant of the hybridoma cell lines are typically screened for antibody binding activity by any one of the methods known in the art such as by enzyme linked immuno sorbent assay (ELISA) or radio immuno assay (RIA). The supernatants are screened for production of mAbs which bind to any of the peptides of the invention (as explained below) or which bind to cells to which they bind, e.g. Daudi cells or T lymphocytes.

DNA sequences which encode any of the amino acid sequences of the heavy chain or light chain of the above mAbs are also encompassed within the scope of the invention. As will no doubt be clear to any man versed in the art, due to the degenerative nature of the genetic code a plurality of nucleic acid sequences may code for the mAb of the invention beyond those shown in Figs. 1 or 2.

The invention also provides expression vectors such as plasmids having said DNA sequences as well as host cells containing one or more of these expression vectors.

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In accordance with another aspect of the invention, there are provided peptidic sequences of a B-cell antigens to which the mAbs of the invention can bind. Searches performed against the non-redundant gene bank database and the EST division determined that these peptidic sequences are novel.

5 In accordance with this additional aspect of the invention there is provided a peptide selected from the group consisting of:

- (a) a peptide having an amino acid sequence as depicted in Fig. 10;
- (b) a peptide having an amino acid sequence as depicted in Fig. 11;
- (c) a peptide having an amino acid sequence as depicted in Fig. 12;
- 10 (d) a peptide having at least 85% identity to any one of the amino acid sequences of the peptides of (a), (b) and (c) above; and
- (e) a protein or a peptide comprising one or more of the peptides of (a)-(d) above.

The peptides of the invention may be used for a variety of diagnostic assays, 15 such as, for example, competitive immuno-assays wherein the level of binding of the mAb of the invention to its native antigen, which exists on T-cells is determined. In addition, the peptides may be used for the production of antibodies in immunized animals which antibodies may then be used for any one of the utilities described above and below.

20 Analogs of all the above peptides also form an additional aspect of the present invention. As will be appreciated by a person versed in the art, the amino acid sequence of the peptides of the invention may be altered, for example, by addition, deletion or conservative or non-conservative substitution of one or more amino acids without substantially altering the antibody binding properties of the 25 peptide

The term "*conservative substitution*" refers to the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physiochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by 30 a standard Dayhoff frequency exchange matrix or BLOSUM matrix. [Six general

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classes of amino acid side chains have been characterized and include: Class I (Cys); Class II (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Trp). For example, substitution of an Asp for another Class III residue such as Asn, Gln, or Glu, is a conservative substitution. The term "*non-conservative substitution*" refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution of an Ala, a Class II residue, with a Class III residue such as Asp, Asn, Glu, or Gln.

The letters used above (and hereinafter) to denote specific amino acids (aa) are in accordance with the 1-letter amino acid symbols recommend by the IUPAC-IUB Biochemical Nomenclature Commission.

Analogues of the above peptides which fall under the scope of the present invention are such which have substantially the same level of binding to the mAbs of the invention as the peptides depicted in Figs. 10-12. The level of binding can be determined by any manner known in the art.

The peptides and analogues of the invention may also be chemically modified and such chemically modified peptides and analogues also form a part of the invention. The term "*chemically modified*" refers to a protein where at least one of its amino acid residues is modified either by natural processes, such as processing or other post-translational modifications, or by chemical modification techniques which are well known in the art. Among the numerous known modifications typical, but not exclusive examples include: acetylation, acylation, amidation, ADP-ribosylation, glycosylation, GPI anchor formation, covalent attachment of a lipid or lipid derivative, methylation, myristylation, pegylation, prenylation, phosphorylation, ubiquitination, or any similar process.

The second finding on which the invention is based is that the mAbs of the invention can bind to a different extent to T-cells obtained from individuals having a malignant disease as compared to the extent of binding of the same mAbs to T-cells of a healthy individual.

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Thus, by a further aspect of the present invention an assay is provided for identifying a tested individual with a high probability of having a malignant disease comprising:

- (a) obtaining a body fluid sample from said individual;
- 5 (b) contacting said sample with at least one mAb of the invention;
- (c) determining the extent of binding of said mAbs to T-cells within said sample; and
- (d) comparing the extent of (c) to the extent of binding of the mAbs of the invention to T-cells in a sample obtained from a healthy individual; a
- 10 significant difference between the above two extents of binding indicating that said tested individual has a high probability of having a malignant disease.

In accordance with the invention, the sample obtained from the individual to be tested may be any body fluid which contains a detectable amount of T-cells. Typically, the body fluid sample is a blood or lymph fluid sample. Preferably,

15 before contacting the mAbs of the invention with the obtained sample, the peripheral blood mononuclear cells (PBMC) in the sample are separated by any one of the methods known in the art such as by Ficoll Hypaque density centrifugation and the separated cells are then contacted with the tested antibodies.

The term "*malignant disease*" in accordance with the invention is to be

20 understood as any kind of malignant disease known in the art at any of its stages.

This term also encompasses malignant diseases which are at their early stages and have not yet elicited clinical symptoms. Preferably this term refers to solid tumors.

The term "*healthy individual*" relates to an individual who does not have a

25 malignant disease, and may also refer to an average level of several individuals or to a level obtained by pooling together body fluids from several individuals. It should be noted that once a standard extent of binding of healthy individuals is established, there is no need to re-establish this standard for every test and the figure established may be used continuously. In accordance with the invention it

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has been found that in healthy individuals about 25% of CD3⁺ T-cells bind to antibodies of the invention.

The term "*high probability*" means that the assay of the invention is an initial screening assay capable of identifying individuals suspected of having a malignant disease. The fact that the individual detected by the method of the invention has indeed a malignant disease will have to be verified later by utilizing additional techniques known in the art.

The term "*extent of binding*" relates to the level of binding of the antibody to an antigen present on the T-cell of the tested individual which extent can be determined by any of the methods known in the art for determining binding levels of antibodies such as ELISA or Western Blotting. The extent of binding may be determined using any detection system such as anti-mouse immunoglobulin or fragments thereof linked to a detectable marker. Examples of such detectable markers are a radioactive group, a fluorescent group, an enzyme capable of catalyzing a reaction yielding a detectable product (such as a color reaction), a biotin group capable of being detected by avidin, etc. By a preferred embodiment, the extent of binding of the mAbs of the invention to the T-cells is carried out by double labeling in which the anti T-cell antibody (e.g. anti-CD3⁺ antibody) is attached to one kind of fluorescent marker and the mAb of the invention is attached to a second type of fluorescent marker. The extent of binding is then determined using fluorescein activated cell sorter (FACS). The quantitation of the extent of binding is achieved by determining the percent of CD3⁺ T-cells (determined by their binding of anti-CD3⁺ antibodies) which also bind the mAb of the invention.

In accordance with the invention, it was found that the total number of CD3⁺ cells in blood samples of individuals having a malignant disease is similar to the number of CD3⁺ cells in blood samples obtained from healthy individuals so that the normalization of the extent of binding of both mAb and CD3⁺ T-cells by using total CD3⁺ binding T-cells both in malignant patients and healthy individuals is valid. However, the percent of the CD3⁺ binding T-cells which also bind the mAb of the invention (hereinafter: "*CD3⁺ mAb cells*") in individuals having a malignant

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disease differs significantly from the percent of CD3⁺ mAb⁺ cells in blood of healthy individuals. The percent of the CD3⁺ mAb⁺ cells in an individual having a malignant disease may either be significantly higher or significantly lower than the percent of CD3⁺ mAb⁺ cells in healthy individuals, depending on the type of the
5 malignant disease.

The extent of binding of a mAb of the invention to a T-cell obtained from a tested individual will be considered to be "*significantly different*" than the extent of binding to T-cells obtained from a healthy individual when the difference in binding of the mAb is statistically different in a significant degree as determined by
10 any of the statistical methods known in the art (e.g. Students t-Test) which are used in connection with results obtained by the experimental methods mentioned herewith.

The invention not only enables to identify individuals having a high probability of having any type of malignant diseases (where the diseased individual
15 has a different extent of binding of T-cells to mAbs of the invention as compared to a healthy individual) but can also help identify individuals having specific types of cancer by determining whether said extent is higher or lower than the corresponding extent in the healthy individual.

Typically, the percent of binding of the mAbs of the invention to T-cells
20 obtained from healthy individuals is in the range of about 25%, i.e. 25% of the cells expressing the CD3⁺ T-cell marker (determined by binding of anti-CD3⁺ antibody to the cells) also bind the mAbs of the invention.

In accordance with the invention, it has been shown that in samples obtained from prostate cancer patients, the percent of CD3⁺ T-cells to which the mAbs of the
25 invention bind are in the range of about 50%.

It was further shown that where the CD3⁺ T-cells originate from samples obtained from colon or breast carcinoma patients, the percent of the cells which also bind to the mAbs of the invention is about 7% and 10%, respectively.

Thus, in accordance with the present invention it has become possible to
30 determine that there is a high probability that there exists a specific type of cancer

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in a body fluid sample taken from a tested individual using a simple and single assay based on the extent of binding of the mAbs of the invention to CD3⁺ cells present in the body fluid sample. The simplicity of the diagnostic assay of the invention which necessitates use of only one kind of mAb to identify an individual
5 having a certain type of cancer is very useful for wide screening of a population.

Thus, the present invention by another of its aspects provides an assay for identifying a tested individual with a high probability of having a specific malignant disease comprising:

- (a) obtaining a body fluid sample from said individual;
- 10 (b) contacting said sample with the mAbs of the invention;
- (c) determining the extent of binding of said mAbs to T-cells in said sample; and
- (d) comparing the extent of binding (c) cells obtained to the extent of binding of the mAbs to T-cells obtained from a healthy individual, the existence of
15 a significant difference in the extents of binding indicating with a high probability that the tested individual has a malignant disease wherein whether the extent of binding to the T-cells from said individual is above or below the extent of the binding of the mAbs in T-cells of healthy individuals, indicates a specific type of malignant disease which the tested individual has with high probability.

20 In particular, where the extent of binding to the mAb of the invention is significantly higher than in healthy individuals the tested individual has a high probability of having prostate cancer.

Where the extent of binding is significantly lower than the healthy individual, the tested individual has a high probability of having colon or breast
25 cancer.

In accordance with the diagnostic aspect of the invention, compositions comprising the mAbs of the invention may be used for diagnosis to identify individuals with the high probability of having a malignant disease (in general) or for identifying a specific malignant disease the individual is likely to have. The
30 invention therefore provides by another of its aspects, a diagnostic composition

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comprising mAbs belonging to at least one of the abovementioned antibodies together with a suitable carrier. The carrier may either be a soluble carrier such as any one of the physiological acceptable buffers known in the art (e.g. PBS) or a solid state carrier such as, for example, latex beads.

5 The present invention also provides kits, e.g. diagnostic assay kits, for utilizing the mAbs of the invention and carrying out the diagnostic assays disclosed above. In one embodiment, the diagnostic kit would conventionally include at least one of the above mAbs in one or more containers, a conjugate of a specific binding partner for the mAb (for example the antigen or analog of the invention), a label
10 capable of producing a detectable signal and directions for its use. The label may be, *a priori*, bound to the monoclonal antibody or, alternatively, the label may be bound to a carrier molecule which then specifically binds to the mAb. The incubation of the tested sample with the diagnostic reagent composition is for a time sufficient to allow binding of the monoclonal antibodies to the cells.

15 By a further aspect of the invention, there are provided pharmaceutical compositions comprising, as an active ingredient, one or more of the mAbs of the invention together. Use of said mAbs for the preparation of pharmaceutical preparations for the treatment of various malignant diseases in an individual is also within the scope of the invention.

20 By yet another aspect the present invention concerns a method of treatment of malignant diseases by administering to an individual in need a therapeutically effective amount of said mAbs. A therapeutically effective amount being an amount capable of alleviating the symptoms of the malignant disease, reducing the symptoms or completely eliminating them.

25 Pharmaceutical compositions comprising the peptides of the invention also constitute an aspect of the invention. Such compositions may be used, for example, for active immunization of an individual to obtain antibodies which may then bind to the T-cells of the individual and elicit an immune response in the individual.

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DETAILED DESCRIPTION OF THE ASPECTS OF THE INVENTION

The main aspects of the invention will now be described with occasional reference to the attached figures. In the following description and figures, the term "BAT antibody" will be used interchangeably with the term "mAbs of the invention".

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the DNA and peptide sequences of the heavy chain variable region of the mAb of the invention;

Figure 2 shows DNA and peptide sequences of the Kappa light chain variable region of the mAb of the invention;

Figure 3 shows an analysis of the amino acid sequence of the heavy chain variable region of the antibody of the invention (designated "BAT"). "BAT" defines the amino acid sequence of the BAT antibody V_H region, while "VMS2" defines the amino acid sequence of the germline VMS2/VGK4 germline gene. Where the BAT sequence and the germline sequence are identical the germline sequence is represented by a dot (.); where mismatches occur the different germline residue is shown. The tables below, the sequence on the following pages describe the frequency with which certain amino acids have been seen at a particular residue position both within the Kabat *et al.*, *Sequences of proteins of immunological interest*, (1991) mouse heavy chain subgroup miscellaneous (Mouse V_H Misc.) and across a larger database of all known mouse V_H sequences (All Mouse V_H);.

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Figure 4 shows an analysis of the amino acid sequence of the kappa light chain variable region of the antibody of the invention (designated in the Fig. As "BAT"). "Mouse" defines the amino acid sequence of the BAT antibody K_K region, while "Germ" defines the amino acid sequence of the germline H4 germline gene. Where the BAT sequence and the germline sequence are identical the germline sequence is represented by a dot (.); where mismatches occur the different germline residue is shown. The tables below and on the following pages describe the frequency with which certain amino acids have been seen at a particular residue position both within the Kabat mouse heavy chain subgroup VI (Mouse V_K VI) and across a larger database of all known mouse V_K sequences (All Mouse V_K);

Figure 5 shows the DNA and peptide sequences of the Kappa light chain variable regions of the chimeric antibody of the invention;

Figure 6 shows the DNA and peptide sequences of the heavy chain variable region of the chimeric antibody of the invention;

Figure 7 shows a schematic representation of the pKN 110 mammalian expression vector used for the expression of the Kappa light chain of the chimeric antibody of the invention;

Figure 8 shows a schematic representation of the pG1D 110 mammalian expression vector used for the expression of the heavy chain of the chimeric antibody of the invention.

Figure 9 shows a graphic representation featuring an example of results of an ELISA assay measuring the binding characteristics of the mouse and the γ 1/Kappa chimeric antibody of the invention to Daudi cells;

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Figure 10 shows the amino acid sequence of peptide 1 of the invention;

Figure 11 shows the amino acid sequence of peptide 2 of the invention;

5

Figure 12 shows the amino acid sequence of peptide 3 of the invention;

Figure 13 is a schematical representation showing the percent of $CD3^{+}$ cells which also bind the mAb of the invention (indicated as "BAT") as compared to the total number of $CD3^{+}$ cells in blood samples of healthy individuals as determined by FACS analysis;.

10

Figure 14 shows the percent of $CD3^{+}$ cells which also bind the mAb of the invention (indicated as BAT) as compared to the total number of $CD3^{+}$ cells in blood samples taken from patients having colon carcinoma as determined by FACS analysis;.

15

Figure 15 shows the percent of $CD3^{+}$ cells which also bind the mAb of the invention (indicated as BAT) as compared to the total number of $CD3^{+}$ cells in blood samples obtained from patients having breast carcinoma;

20

Figure 16 shows the percent of $CD3^{+}$ cells which also bind the mAb of the invention (indicated as BAT) as compared to the total number of $CD3^{+}$ cells in blood samples obtained from patients having prostate carcinoma;.

25

Figure 17 is a schematic representation showing the mean percent of $CD3^{+}$ cells which bind the mAb of the invention (indicated as BAT) in healthy individuals as compared to patients having breast carcinoma, colon carcinoma or prostate carcinoma;.

30

Figure 18 is a photograph of a Western Blot of peptides obtained from T-cells of individuals having prostate cancer, ear, nose and throat (ENT) carcinoma, breast carcinoma or from membranes of Daudi cells. The Blot was incubated with the mAb of the invention and shows an increased amount of antigen in T-cells obtained from patients having prostate carcinoma as compared to an undetectable level of antigen in T-cells obtained from patients having breast carcinoma;.

10

I. SEQUENCING OF THE MAB

(A) Abbreviations

Fetal Calf Serum (FCS); ribonucleic acid (RNA); messenger RNA (mRNA); deoxyribonucleic acid (DNA); copy DNA (cDNA) ; polymerase chain reaction (PCR); minute (min); second (sec); Tris-borate buffer (TBE).

15

(B) Materials

Media components and all other tissue culture materials were obtained from Life Technologies (UK). The RNA isolation kit was obtained from Stratagene (USA) while the 1st strand cDNA synthesis kit was purchased from Pharmacia (UK). All the constituents and equipment for the PCR-reactions, including AmpliTaq® DNA polymerase, were purchased from Perkin Elmer (USA). The TA Cloning® kit was obtained from Invitrogen (USA). Agarose (UltraPure™) was obtained from Life Technologies (UK). The Thermo Sequences™ pre-mixed cycle sequencing kit and the Vistra 725 DNA sequencing machine were both purchased from Amersham (UK). All other molecular biological products were obtained from New England Biolabs (USA).

25

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(C) Experimental techniques: PCR cloning and sequencing of the mouse BAT antibody variable region genes

The mouse BAT hybridoma cell line and the Daudi cell line were
5 successfully transferred to the MRC-CC and both cell lines were grown, in
suspension, using RPMI (without glutamine) supplemented with 10% (v/v) FCS,
100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, 1 mM
sodium pyruvate and 12.5 units/ml Nystatin.

Approximately 10^8 of viable cells of the BAT hybridoma cell line were
10 harvested and, from the 10^8 cells, total RNA was isolated using an RNA Isolation
kit according to the manufacturers instructions. The kit used a guanidinium
thiocyanate phenol-chloroform single step extraction procedure as described by
Chomczynski and Sacchi, *Anal. Biochem.*, **162**:156, 1987. Also following the
manufacturers instructions a 1st Strand cDNA synthesis kit was employed to
15 produce a single-stranded DNA copy of the BAT hybridoma mRNA using the
NotI-(dT)₁₈ primer supplied in the kit. Approximately 5 µg of total RNA was
used in each 33 µl final reaction volume. The completed reaction mix was then
heated to 90°C for 5 min. to denature the RNA-cDNA duplex and inactivate the
reverse transcriptase, before being chilled on ice.

20 To PCR-amplify the mouse heavy chain variable region gene (V_H gene)
and the mouse kappa light chain variable region gene (V_K gene) from the
hybridoma cell line the method described by Jones and Bendig, *Bio/Technology*,
9:8, 1987 was followed. Essentially, two series of degenerate primers, one
designed to anneal to the leader sequences of the mouse heavy chain genes (i.e.
25 MHV1-12; Table 1) and one designed to anneal to the leader sequences of mouse
kappa light chain genes (i.e. MKV1-11; Table 2) were used, in conjunction with
primers designed to anneal to the 5'-end of the appropriate constant region gene,
to PCR-clone the murine variable region genes.

Separate PCR-reactions were prepared for each of the degenerate primers
30 with their appropriate constant region primer, in a special PCR-room using

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specific protocols designed to minimize the possibility of cross-contamination. Amplitaq® DNA polymerase was used to amplify the template cDNA in all cases. The PCR-reaction tubes were then loaded into a Perkin Elmer 480 DNA thermal cycler and cycled (after an initial melt at 94°C for 1.5 min) at 94°C for 1 min and 72°C for 1 min over 25 cycles. At the completion of the last cycle a final extension step at 72°C for 10 min was carried out before the reactions were cooled to 4°C. Except for between the annealing (50°C) and extension (72°C) steps, when an extended ramp time of 2.5 min was used, a 30 sec ramp time between each step of the cycle was employed.

10 10 µl aliquots from each PCR-reaction were run on a 1% agarose/TBE (pH 8.8) gel to determine which had produced a PCR-product of the correct size. Those PCR-reactions that did appear to amplify full-length variable region genes were repeated to produce independent PCR-clones and thereby minimize the effect of PCR-errors. 1-6 µl aliquots of those PCR-products of the correct size
15 were directly cloned into the pCRII™ vector, provided by the TA Cloning® kit, and transformed into INA αF' competent cells as described in the manufacturers instructions. Colonies containing the plasmid, with a correctly sized insert, were identified by PCR-screening the colonies using the pCRII Forward and pCRII Reverse oligonucleotide primers described in Table 3 below according to the
20 method of Güssow and Clackson, *Nucleic Acids Res.*, 17:4000, 1989

Those putative positive clones identified were double-stranded plasmid DNA sequenced using the Vistra DNA sequencing machine and the Thermo Sequenase™ pre-mixed cycle sequencing kit as described in the manufacturers instructions.

25

Example 1: Cloning and sequencing of the heavy chain variable region of the BAT antibody

As with all humanization projects, a strict PCT-cloning and sequencing
30 protocol was followed. This was done to minimize the possibility of introducing

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errors into the wild-type sequences of the mouse V_H variable region genes from the BAT hybridoma cell line. Only if all the DNA sequence data from at least two different V_H gene clones, from the hybridoma cell line expressing the murine BAT antibody, matched perfectly were the gene sequences accepted as correct.

5 Three separate PCR-products, each from a different total RNA preparation and subsequent first strand cDNA synthesis reaction, were PCR-cloned and completely DNA sequenced on both strands. Although all twelve heavy chain primers were tested (Table 1), only the MHV9 primer (in conjunction with MHCG3 – designed to anneal to the CH_1 domain of the mouse $\gamma 3$ heavy chain
10 gene) was PCR-amplified an approximately 460 bp product which was then TA-cloned into the pCRII™ cloning vector (data not shown).

DNA sequence analysis of several individual clones from each of the three PCR-products (each from different 1st strand synthesis reactions and subsequent PCR-reactions) resulted in the determination of the BAT antibody heavy chain
15 variable region sequence as described in Fig. 1. This sequence was confirmed on both DNA strands for all three PCR-clones studied.

Example 2: Cloning and sequencing of the kappa light chain variable region of the BAT antibody

20

The single stranded cDNA template, produced via 1st strand synthesis, was PCR-amplified using a series of kappa light chain degenerate primers (Table 2 below). However, this resulted in the amplification of a number of PCR-products from more than one degenerate primer, suggesting that more than one variable
25 region gene was being transcribed, at least, by the BAT hybridoma cell line.

First, a PCR-product was seen when the MKV2 primer (which, like all of the MKV series of primers, anneals to the 5' end of the DNA sequence of the kappa light chain signal peptide) and MKC (which is designed to anneal to the 5' end of the mouse kappa constant region gene) were used together. Previous
30 in-house experience had shown us that the MKV2 primer would PCT-amplify an

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aberrant mRNA transcript. This aberrant pseudogene was present in all standard fusion partners derived from the original MOPC-21 plasmacytoma cell line and was known as MOPC-21n Deyev, S.M., *et al.*, *Genetica*, **85**:45, 1991. NO-0 was a cell line which was derived from MOPC-21 line, and it was this line which was
5 used as the fusion partner to produce the BAT hybridoma. Consequently, it was not surprising that a PCR-product was seen when using the MKV2 primer. This product was analyzed and shown to be the non-functional pseudogene (data not shown).

Unusually, another pseudogene, previously identified as being secreted by
10 the related cell line NS-1 Hamlyn, P.H., *et al.*, *Nucl. Acis Res.*, **9**:4485, 1981 and normally PCR-cloned when using the MKV7 primer in conjunction with MKC primer, was not seen in any of the PCR- products so far analyzed. Since the NS-1 and NS-0 cell lines were very closely related, this was a little surprising. However, it also highlighted the confusing nature of kappa light chain
15 transcription that was present in the BAT hybridoma cell line.

Another PCR-clone, which ultimately turned out to be the V_{κ} gene of the BAT antibody, was also successfully PCR-amplified from the BAT hybridoma cell line with the primers MKV5 and MKC. Following transformation of the approximately 450 bp product into $INV_{\alpha}F'$ competent cells, putative positive
20 transformants were identified using the PCR-screening assay and then DNA sequenced.

From sequence analysis of two individual clones of the MKV5 product (each from different 1st strand synthesis reactions and subsequent PCR-reactions) the DNA sequence of the BAT antibody kappa light chain variable region gene
25 was determined (Fig. 2). This sequence was again confirmed on both DNA strands for each clone.

Example 3 Sequence analysis of the mouse BAT antibody variable regions

The amino acid sequence of the BAT V_κ and V_H regions were compared to the consensus sequences of murine variable region subgroups that were defined in the Kabat (*Supra*) database. From this analysis the BAT V_H region was found to most closely match the consensus sequence of mouse kappa subgroup VI. Similar comparisons of the BAT V_H region to the Kabat database found that it exhibited the closest match to the consensus sequence of mouse heavy chain subgroup "miscellaneous".

A comparison of the above BAT antibody variable region sequences to a database of murine germ lines, found that the closest germline gene to the BAT V_H gene was VMS/VGK4 (Fig. 3), whilst the closest germline gene to the BAT V_κ gene was H4 (Fig. 4). As can be seen in Fig. 3, those mismatches that did occur between the BAT V_H gene and its closest germline gene were, unsurprisingly, predominantly located in the CDR2 and CDR3. There were only three framework changes, and all these were located in FR3. With respect to the BAT V_κ gene (Fig. 4), it was again not all together surprising that the majority of mismatches were positioned in the CDRs. The four differences that were located in the FRs were all highly conservative changes, except for the cysteine at position 72 (Kabat numbering) in FR3. Its location immediately adjacent to an important canonical residue (position 71) suggested that the cysteine may have been playing a key role in antigen binding. However, only through modeling the Fv domain could such a supposition be clarified.

Nevertheless, these analyses confirmed that both the V_H regions and the V_κ regions of the mouse BAT variable regions appeared to be typical of mouse variable regions.

- 20 -

Table 1 PCR-primers used in the cloning of the BAT heavy chain variable region gene

5	Name	Sequence (5' → 3')
	MHV5 ^a (30 mer)	ATGGACTCCAGGCTCAATTTAGTTTTCTT
	MHV9 ^a (30 mer)	ATGGATTGGGTGTGGACCTTGCTATTCCTG
		C A
10	MHCG3 ^b (21 mer)	CAAGGGATAGACAGATGGGGC

^a MHV indicates a primer that hybridizes to leader sequences of mouse heavy chain variable region genes.

^b MHCG indicates primers that hybridize to mouse constant region genes.

15

Table 2 PCR-primers used in the cloning of the BAT kappa light chain variable region gene

20

	Name	Sequence (5' → 3')
25	MKV2 ^a (30 mer)	ATGGAGACAGACACACTCCTGCTATGGGTG
		T T
	MKV5 ^a (30 mer)	ATGGATTTTCAGGTGCAGATTATCAGCTTC
		A T
30	MKV6 ^a (30 mer)	ATGAGGTGCCCTGTTCAGTTCCTGGGG
		T TT C G C T A
	MKV11 ^a (30 mer)	ATGGAAGCCCCAGCTCAGCTTCTCTCC
	MKC ^b (20 mer)	ACTGGATGGTGGGAAGATGG

35

^a MKV indicates primers that hybridize to leader sequences of mouse kappa light chain variable region genes

^b MKC indicates the primer that hybridizes to the mouse kappa constant region gene

40

Table 3 Primers for PCR screening transformed colonies

5	Name	Sequence (5' → 3')
	pCRII Forward Primer (18 mer)	CTAGATGCATGCTCGAGC
	pCRII Reverse Primer (21 mer)	TACCGAGCTCGGATCCACTAG

10

II. CONSTRUCTION AND EXPRESSION OF THE CHIMERIC ANTIBODY OF THE INVENTION

(A) Abbreviations

15 The following non-SI unit and other abbreviations were used:

Polymerase chain reaction (PCR); deoxyribonucleic acid (DNA); copy DNA (cDNA); kappa light chain variable region (V_K); heavy chain variable region (V_H); minute (min); Tris-borate buffer (TBE); phosphate buffered saline (PBS); room temperature (RT), bovine serum albumin (BSA); hydrochloric acid (HCl); horseradish peroxidase (HRP); low fat milk LFM); hour (hr); percent (%);
 20 O-phenylenediamine dihydrochloride (OPD); multiple cloning site (MCS).

(B) Materials

Media components and all other tissue culture materials were obtained
 25 from Life Technologies (UK). The constituents for the PCR-reactions, including AmpliTaq® DNA polymerase, were purchased from Perkin Elmer (USA). However, the TA Cloning® kit and INVαF' competent cells were obtained from Invitrogen (USA). DH5α competent cells and agarose (UltraPure™) were obtained from Life Technologies (UK). The Thermo Sequenase™ pre-mixed
 30 cycle sequencing kit and the Vistra 725 DNA sequencing machine were both purchased from Amersham (UK). The Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit used with the ABI Prism 310 Genetic Analyzer were

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purchased from PE Applied Biosystems (UK). All other molecular biological products described were obtained either from New England Biolabs (USA) or Promega (USA). Nunc-Immuno Plate MaxiSorp™ immunoplates were purchased from Life Technologies (UK) while the Corning easy wash ELISA plates were
5 obtained from Corning Laboratory Sciences Company (UK). The goat anti-human IgG (Fc_γ fragment specific) antibody, the goat anti-human kappa light chain/HRP conjugate and the AffinPure goat anti-human IgG (Fc_γ fragment specific)/HRP conjugate were obtained from Jackson ImmunoResearch Laboratories Inc. (USA). K-Blue TMB substrate and Red Stop solution were
10 purchased from Neogen Inc. (USA). All other products for the ELISA were obtained from Sigma (UK). Microplate Manager® data analysis software package was purchased from Bio-Rad (UK). The micro-volume stirred ultrafiltration cell and PM30 filter membrane were obtained from Amicon PLC (UK), while the Immunospecific® (G) IgG purification kit was purchased from
15 Pierce PLC (UK).

(C) Experimental Techniques

C1 Construction of chimeric $\gamma 1/\kappa$ BAT antibody

The previously isolated mouse kappa light chain variable region (V_κ) gene
20 (Fig. 1) and heavy chain variable region (V_H) gene (Fig. 2) were modified at the 5'- and 3'-ends, using specifically designed PCR-primers (Table 1), to enable expression of the BAT variable region genes in mammalian cells as part of a chimeric mouse-human antibody. To achieve this separation PCR-reactions were prepared for each variable region gene in a specific PCR-room using specific
25 protocols designed to minimize the possibility of cross-contamination. The plasmids BATV_H-pCR2.1 and BATV_κ-pCR2.1 were used as templates and AmpliTaq® DNA polymerase was used to amplify these templates. Primers B8814 and B8815 (Table 4) were used to PCR-modify the BAT V_H gene while primers C0224 and C0225 (Table 4) were used to PCR-mutate the BAT V_κ gene.

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The PCR-reaction tubes were cycled (after an initial melt at 94°C for 3 min) at 94°C for 50 s, 72°C for 1 min 30 s over 30 cycles. At the completion of the last cycle a final extension step at 72°C for 10 min was carried out before the reactions were cooled on ice. 5 µl aliquots from each PCR-reaction were then run
5 on a 1.2% agarose/TBE (pH 8.8) gel to determine which had produced a PCR-product of the correct size.

1-2 µl aliquots of those PCR-products of the correct size were directly cloned into the pCR2.1™ vector, provided by the TA Cloning® kit, and transformed into INVαF' competent cells as described in the manufacturers
10 instructions. Colonies containing the plasmid, with a correctly sized insert, were identified by PCR-screening the colonies using the 1212 and 1233 oligonucleotide primers (Table 5) according to the method of Güssow and Clackson (*Supra*) Those putative positive clones identified were double-stranded plasmid DNA sequenced using both the Vistra DNA sequencing machine and
15 ABI Prism 310 Genetic Analyzer. The Thermo Sequenase™ pre-mixed cycle sequencing kit and the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit were used as described in the manufacturers instructions with the primers 1212 and 1233 (Table 5).

Those clones containing the correctly adapted BAT V_K and V_H genes
20 (Figs. 5 and 6, respectively) were subcloned, as a *Hind*III-*Bam*HI fragments, into the expression vectors pKN110 (Fig. 7) and pG1D110 (Fig. 8), respectively, to express chimeric light and heavy chains in mammalian cells. The ligated expression vectors (i.e. pKN110-BATV_K and pG1D110-BATV_H) were then transformed into DH5α competent cells. Positive clones, containing the correctly
25 constructed expression vectors, were finally identified by restriction digest analysis.

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C2 Co-transfection of chimeric $\gamma 1/\kappa$ BAT antibody vector DNA into COS cells

The method of Kettleborough *et al.* was followed to transfect the
5 mammalian expression vectors into COS cells. Briefly, the DNA (10 μ g each of
the kappa light chain expression vector pKN110-BATV κ and heavy chain
expression vector pG1D110-BATV H) was added to a 0.70 ml aliquot of 1×10^7
cells/ml in PBS and pulsed at 1900 V, 25 μ F capacitance using a Bio-Rad Cene
Pulser apparatus. Following a 10 min recovery at RT the electroporated cells
10 were added to 8 ml of DMEM containing 5% FCS and incubated for 72 hr in 5%
CO $_2$ at 37°C. After 72 hr incubation, the medium was collected, spun to remove
cell debris and analyzed by ELISA for chimeric BAT antibody production.

C3 Quantification of chimeric $\gamma 1/\kappa$ antibody via ELISA

15 Each well of a 96-well Nunc-Immuno Plate MaxiSorpTM immunoplate as
first coated with 100 μ l aliquots of 0.4 ng/ μ l goat anti-human IgG (Fc γ fragment
specific) antibody, diluted in PBS and incubated overnight at 4°C and removed
prior to use. 100 μ l/well aliquots of the experimental samples (i.e. harvested COS
cell supernatants – spun to remove cell debris) and 1:2 sample dilutions, diluted
20 in sample-enzyme conjugate buffer (0.1 M Tris-HCl (pH 7.0), 0.1 M NaCl,
0.02% (v/v) TWEEN-20 and 0.2% (w/v) BSA), were then dispensed onto the
immunoplate. In addition, a purified human $\gamma 1/\kappa$ antibody (1000 ng/ μ l), which
was used as a standard and serially diluted 1:2, and also loaded onto the
immunoplate. The immunoplate was incubated at 37°C for 1 hr before being
25 washed with 200 μ l/well of wash buffer (PBS/0.1% (v/v) TWEEN-20) three
times. 100 μ l of goat anti-human kappa light chain/horseradish peroxidase
conjugate, diluted 5000-fold in sample-enzyme conjugate buffer, was added to
each well, following which the immunoplate was incubated at 37°C for 1 hr
before it was washed as before. 150 μ l aliquots of K-Blue substrate were then
30 added to each well, following which the immunoplate was incubated for 10 min

- 25 -

at RT in the dark. The reaction was finally halted by dispensing 50 μ l of Red Stop into each well. The optical density at 655 nm was then determined using a Bio-Rad 3550 microplate reader in conjunction with the Microplate Manager® software package.

5

C4 Purification of the chimeric BAT antibody

The chimeric BAT γ 1/ κ antibody was purified from COS cell supernatants in two stages. First, a micro-volume stirred ultrafiltration cell with a PM30 filter membrane was used, according to the manufacturers instructions, to reduce the
10 volume of the raw, non-purified supernatant. Then an Immunopure® (G) IgG purification kit was used to affinity purify the chimeric BAT antibody from the concentrated supernatant, also according to the manufacturers instructions.

C5 Daudi cell ELISA

15 The cell ELISA assay was carried out using the Daudi cell cultured from an original stock also by Dr. Hardy (Felsenstein Medical Research Center, Rabin Medical Center, Beilinson Campus, Petach Tikva, 49100, Israel). Minor modifications were made to the assay depending upon whether the mouse or the mouse-human chimeric BAT antibody was being analyzed. When assaying the
20 binding affinity of the mouse BAT antibody a goat anti-mouse IgG (Fab specific)/HRP conjugate (diluted 1:15000) was used as the secondary antibody. Conversely, when measuring the affinity of the chimeric BAT antibody AffiniPure goat anti-human IgG (Fc_γ fragment specific)/HRP conjugate (diluted 1000-fold) was used.

25 The Daudi cells (2 days after being passaged) were first plated at 10^5 cells/well in a 96 well Corning easy wash ELISA plate and then incubated overnight at 37°C in a dry incubator. The next day, 200 μ l of rehydration buffer (PBS containing 10% FCS and 0.05% azide) was added to each well which was then left for a minimum of 1 hr. The rehydration buffer was then decanted off

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before 50 μ l aliquots of various 1:2 serial dilutions of the purified BAT antibody was added to the wells of the plate. The plate was again incubated overnight (at 4°C), washed twice with 200 μ l/well of PBS containing 5% LFM and allowed to dry. 50 μ l/well of the HRP conjugated secondary antibody was then added before
5 a series of six different washes (i.e. one wash with PBS containing 5% LFM, three washed with the same buffer supplemented with 0.05% TWEEN-20, followed by a further two washes with the PBS/LFM buffer) were carried out. 200 μ l/well of 0.4 mg/ml OPD substrate in 0.05 M citrate buffer (pH 5.0) and 60 mg/ml hydrogen peroxide was then added before the ELISA plate was incubated
10 in the dark and at RT until the color had developed (usually about 30 min). Finally, the reaction was stopped by the addition of 50 μ l/well of 2.5 M sulfuric acid and the optical density at 490 nm was then measured using a Bio-Rad 3550 microplate reader in conjunction with the Microplate Manager® software package.

15

Results

Example 4 Construction of the chimeric $\gamma 1/\kappa$ BAT antibody

As with all projects, a strict PCR-cloning and sequencing protocol was followed. This was done to minimize the possibility of introducing errors into the
20 wild-type sequences of the mouse variable region genes during the PCR-modification step. Using the primers C0224 and C0225 (Table 1) the mouse BAT V_{κ} gene (Fig. 2) was modified via PCR to produce a 418 bp band (data not shown). This PCR-product was ligated into the pCR2.1 plasmid and transformed into INV α F' competent cells. Similarly, the mouse BAT V_H gene (Fig. 1) was
25 PCR-mutated using primers B8814 and B8815 (Table 1) to produce a 436 bp band (data not shown). This PCR-product was also ligated into the pCR2.1 plasmid and transformed into INV α F' competent cells.

Putative positive transformants were then detected using the PCR-screening assay (data not shown) before finally being ds-DNA sequenced

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on the ABI Prism 310 Genetic Analyzer. Figs. 3 and 4 show the results of this DNA sequence analysis of the chimeric BAT V_κ gene and BAT V_H gene, respectively. The analysis was carried out both to confirm their successful mutagenesis and also show the presence of any PCR-errors that may have been introduced into the genes. Only one PCR-reaction was actually carried out for each variable region gene and only two clones from each of these PCR-reactions were eventually DNA sequences to completed.

Nevertheless, this proved sufficient to isolate at least one clone for each modified variable region gene which contained the correct modified DNA sequence.

The mutated V_H and V_κ genes were then subcloned into the appropriate expression vectors, as *hindIII/BamHI* fragments, to create pKN110-BATV_κ (7.88 kb) and pG1D110-BATV_H (7.55 kb), respectively. The fidelity of the expression vectors constructed was then confirmed via restriction enzyme analysis (data not shown). Once co-transfected into COS cells, these vectors would allow the transient expression of a γ1/κ version of the chimeric BAT antibody.

In addition, as an extra component to the BAT antibody humanization project, the BAT V_H gene was also subcloned, as a *HindIII/BamHI* fragment, into both the pG3D110 and the pG4D1100 heavy chain expression vectors. These vectors were identical to pG1D110, save for the replacement of the cDNA copy γ1 human constant region genes with either a cDNA copy of the 3γ constant region genes (in the case of pG3D110) or the cDNA of the γ3 constant region genes (in the case of pG3D110) or the cDNA of the γ4 constant region genes (in the case of pG3D110). The construction of these vectors (i.e. pG3D110-BATV_κ, of both γ3/κ and γ4/κ versions of the chimeric BAT antibody in COS cells.

Example 5 Transient expression of the chimeric γ1/κ BAT antibody

The two vectors pKN110-BATV_κ and pG1D110-BATV_H were co-transfected into COS cells in a series of repeated transient expression

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experiments. After being expressed for 72 hr the mouse-human $\gamma 1/\kappa$ chimeric BAT antibody was detected in the supernatant of the COS cell co-transfections via the $\gamma 1/\kappa$ ELISA. From these assays the mean concentration of $\gamma 1/\kappa$ chimeric BAT antibody detected in the media was calculated to be 509 ± 272 ng/ml.

5 Interestingly, the $\gamma 3/\kappa$ and $\gamma 4/\kappa$ versions of the chimeric BAT antibody appeared to produce significantly greater quantities of antibody following their expression COS cells. Specifically, when pG3D110-BATV_H and pKN110BATV _{κ} were co-transfected into COS cells, initial analysis of the supernatant (using the ELISA method described in Section 4.3 and human IgG3/kappa antibody as a
10 standard) measured the expression levels of the chimeric $\gamma 3/\kappa$ BAT antibody to be 6.7 μ g/ml. Moreover, when pG4D110-BATV_H pKN110-BATV _{κ} were expressed in COS cells, the same ELISA (using human IgG4/kappa antibody as a standard) measured the expression levels of the chimeric $\gamma 4/\kappa$ BAT antibody to be 8.2 μ g/ml.

15

Example 6 Purification of the chimeric $\gamma 1/\kappa$ BAT antibody

Harvesting approximately 8 ml per co-transfection, a series of transfections were carried out until 200 ml of COS supernatant had been collected. The volume of this supernatant was then reduced to 15 ml by passing
20 the supernatant through a micro-volume stirred ultrafiltration cell with a PM30 filter membrane – which had a molecular weight cut-off of 30 kDa.

The Immunopure® (G) IgG purification kit essentially comprised of a 2 ml column of immobilized Protein G column. The antibody was eluted from the column with 6 ml of elution buffer, the eluate of which was collected in 1 ml
25 fractions. The concentration of chimeric $\gamma 1/\kappa$ BAT antibody in each fraction was then assayed using the ELISA method described in Section C3. This analysis found that the chimeric antibody was present in Fraction 3 (42.05 μ g/ml) and Fraction 4 (20.05 μ g/ml), which correspond to a total recovery of 62.1 μ g of

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chimeric $\gamma 1/\kappa$ BAT antibody. This was stored at -20°C , until its subsequent transfer to Curetech for further analysis.

Example 7 Analysis of Daudi cell binding by the chimeric $\gamma 1/\kappa$ Bat antibody

Using the Daudi cell ELISA it was clearly shown that the purified chimeric $\gamma 1/\kappa$ BAT antibody bound to Daudi cells. Fig. 9 shows a typical example of one experiment. However, what was less conclusive was the binding of similar concentrations of mouse BAT antibody, in the same ELISA, which appeared to be lower than the chimeric antibody. Nevertheless, since the conjugated secondary antibody used to detect antibody binding to the Daudi cells was different for each antibody construct, no direct comparison of the binding of the two versions can legitimately be made.

Table 4 Primers used to PCR-modify the mouse BAT antibody kappa light chain and heavy chain variable region genes to allow their expression as part of a chimeric $\gamma 1/\kappa$ BAT antibody in mammalian cells

Name	Sequence (5' → 3')
C0225 (42 mer)	CCCAAGCTTGCCGCCACCATG GATTTTCAGGTGCAGATTATC
C0224 (39 mer)	CGCGGATCCACTCACGTTTTA TTTCCAACCTTTGTCCCCG
B8815 (40 mer)	GGATCCACTCACCTGAGGAGA CGGTGACTGAGGTTCCTTG
B8814 (42 mer)	AAGCTTGCCGCCACCATGGCT TGGGTGTGGACCTTGCTATTC

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Table 5 Primers used to PCR screen the transformed colonies and DNA sequence the PCR-modified variable region genes of the BAT antibody

5	Name	Sequence (5' → 3')
	Huγ1 (17 mer)	TTGGAGGAGGGTGCCAG
	HCMVi.3s (28 mer)	GTCACCGTCCTTGACACGCGT
10		CTCGGGA
	FOR (18 mer)	TGTAAACGACGGCCAGT
	REV (18 mer)	GAAACAGCTATGACCATG
15	B6990 (27 mer)	CAGCATATGTTGACTCTCCAC
		TGTCGG
	B6991 (27 mer)	GTCAACATATGCTGAAGAGTT
20		CAAGGG
	B8809 (18 mer)	TGCCAGGTCAAGTGTAAG
	B8810 (18 mer)	AAGCCAGGTTGGATGTCC

25

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IV AMINO ACID SEQUENCES OF 3 PEPTIDES TAKEN FROM THE DAUDI B-CELL LYMPHOBLASTOID CELL LINE ANTIGEN TO WHICH THE MABS OF THE INVENTION BIND

5 Three peptides comprised in the antigenic epitope of the Daudi B lymphoblastoid cells to which the mAbs of the invention bind were sequenced. Their sequence depicted in Figs. 10, 11 and 12.

Searches performed against the non-redundant gene bank database and the EST Division yielded no hits when the three peptides were ran as queries using
10 the TBLASTN algorithm (Version 2) with an EXPECT value of 10 and the matrix BLOSUM 62.

However, since the peptides are small peptides, they were submitted again at a higher EXPECT value to make the search less stringent. The filter was also unmasked for low complexity which can eliminate potentially confounding
15 matches (e.g. hits against proline-rich regions or proly-A tails) from the blast reports, leaving regions whose blast statistics reflect the specificity of their pairwise alignment. The three peptides of the invention did not yield any hit with the gene bank and the EST division database even at a very low stringency.

Thus, in accordance with the above results, the three above peptides seem
20 to be novel peptides.

IV DIAGNOSIS OF MALIGNANT DISEASES IN PATIENTS USING THE MAB OF THE INVENTION

25 Peripheral blood lymphocytes from tested individuals were double-labeled using the anti-CD3 antibody and one of the mAbs of the invention. The percent of CD3⁺ cells which bind the mAbs of the invention were determined. In accordance with the invention, it has been shown that the number of the CD3⁺mAb⁺ cells in individuals having a malignant disease differs from the
30 percent of these cells in blood samples obtained from healthy individuals. The fact that there exists a significant difference of the percent of the CD3⁺ cells in

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the individuals having a malignant disease and whether the difference is above or below the percent of $CD3^+ mAb^+$ cells obtained from healthy individuals enables to determine at high probability whether the individual has a malignant disease as well as the specific kind of malignant disease which the tested individual may
5 have.

Typically, human peripheral blood lymphocytes were obtained from 20 ml blood of either a healthy individual or from cancer patients by Ficoll Hypaque density centrifugation. The cells were washed and suspended in PBS containing 0.5% BSA and 0.05% as acid. The samples containing 0.5×10^6 cells were used
10 for FACS analysis. First, the cells were incubated with a saturated amount of the mAb of the invention for 45 mins. at $0^\circ C$ followed by their incubation with an anti-mouse mAb conjugated to FITC for 30 mins. on ice. After two washes and centrifugation at 1200 rpm cells were incubated with an anti-human CD3 conjugated to PE antibodies for 30 mins. on ice. Following this incubation, the
15 cells were washed twice and the sample is analyzed by a FACS scan (Bectan Dickinson). The results are shown in Figs. 13 to 17.

As can be seen in Fig. 13, as well as in Fig. 17, the percent of $CD3^+ BAT^+$ cells (as compared to total $CD3^+$ cells) in blood samples obtained from healthy individuals is in the range of about 25%. As seen in Fig. 14, the percent of the
20 $CD3^+ BAT^+$ cells in blood samples obtained from patients having colon carcinoma is substantively lower, as compared to healthy individuals, in the range of about 7%. Similarly, the percent of $CD3^+ BAT^+$ cells in blood samples obtained from patients having breast carcinoma was in the range of about 10% (Fig. 15). These results clearly indicate that colon and breast carcinoma can be
25 identified by the fact that the percent of $CD3^+ BAT^+$ cells is lower as compared to healthy individuals.

The percent of $CD3^+ BAT^+$ cells in blood samples obtained from prostate carcinoma patients is significantly higher than the percentage in blood samples of healthy individuals as seen in Fig. 16 and is in the range of about 50%. These
30 results clearly indicate that prostate carcinoma can be identified by the fact that

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the percent of CD3⁺ BAT⁺ cells is higher a compared to healthy individuals. As seen in Fig. 18, the amount of the antigen to, which the mAb of the invention bind found on T-cells, obtained from prostate carcinoma patients is very high while the antigen is undetectable in T-cells obtained from patients of breast carcinoma.

The above results show that the mAbs of the invention may be used in order to identify an individual suffering from a certain kind of malignant disease. Thus, if a blood sample is obtained from a tested individual and the extent of binding of the mAbs of the invention to CD3⁺ cells in the sample is significantly high (in the range of about 50%), there is a very high probability that the tested individual is suffering from prostate cancer. Against this, if the percent of the CD3⁺ cells in the sample is significantly low as compared to healthy individuals (in the range of about 7% or 10%), there is a high probability that the tested individual is suffering from breast or colon carcinoma. Obviously, if the tested individual is a male individual, there is a high probability of his suffering from colon carcinoma.

The above examples are not to be construed as limiting and additional correlations between the percent of CD3⁺ cells which bind the mAbs of the invention and other malignant diseases are also within the scope of the invention.

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CLAIMS:

1. A monoclonal antibody having a variable region selected from the group consisting of:
 - (a) a monoclonal antibody having a heavy chain variable region
5 comprising the amino acid sequence of Fig. 1;
 - (b) a monoclonal antibody having a Kappa light chain variable region comprising the amino acid sequence of Fig. 2;
 - (c) a monoclonal antibody having a heavy chain variable region comprising the amino acid sequence of Fig. 1 and the Kappa light chain variable
10 region comprising the amino acid sequence of Fig. 2;
 - (d) a monoclonal antibody having a heavy chain variable region having at least 70% identity to the amino acid sequence of Fig. 1;
 - (e) a monoclonal antibody having a light chain variable region having at least 70% identity to the amino acid sequence of Fig. 2.
- 15 2. A monoclonal antibody according to Claim 1, having a heavy chain variable region comprising the amino acid sequence of Fig. 1.
3. A monoclonal antibody according to Claim 1, having a kappa light chain variable region comprising the amino acid sequence of Fig. 2.
4. A monoclonal antibody according to Claim 1, having a heavy chain variable
20 region comprising the amino acid sequence of Fig. 1 and the Kappa light chain variable region comprising the amino acid sequence of Fig. 2.
5. A monoclonal antibody according to Claim 1, having a heavy chain variable region having at least 70% identity to the amino acid sequence of Fig. 1.
6. A monoclonal antibody according to Claim 1, having a light chain variable
25 region having at least 70% identity to the amino acid sequence of Fig. 2.
7. An antibody binding to an antigen to which any one of the mAbs of Claims 1-6 specifically bind.
8. An antibody according to any of Claims 1-7, being a chimeric human-mouse antibody.

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9. A nucleic acid sequence encoding the amino acid sequence of any of the mAbs of Claims 1-8.
10. An expression vector having the nucleic acid sequence of Claim 9
11. An expression vector according to Claim 10, being designated as plasmid
5 pKN110.
12. An expression vector according to Claim 10 being designated as plasmid pG1D110.
13. A cell transfected with any one of the expression vectors of Claims 10 to 12.
14. A hybridoma cell line producing any of the mAbs of Claims 1 to 8.
- 10 15. A peptide having the amino acid sequence of the peptide depicted in Fig. 10.
16. A peptide having the amino acid sequence of the peptide depicted in Fig. 11.
17. A peptide having the amino acid sequence of the peptide depicted in
15 Fig. 12.
18. A peptide having at least 85% identity to the amino acid sequences of any one of the peptides of Claims 12 to 14.
19. A protein or peptide comprising one or more of the peptides of Claims 12 to 15.
- 20 20. An analog of any of the peptides of Claims 12 to 15, having substantially the same level of binding to any one of the mAbs of Claim 1.
21. An assay for identifying a tested individual with a high probability of having a malignant disease comprising:
 - (a) obtaining a body fluid sample from said individual;
 - 25 (b) contacting said sample with at least one of the mAbs of Claims 1 to 8;
 - (c) determining the extent of binding of said mAbs to T-cells within said sample; and

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(d) comparing the extent of (c) to the extent of binding of the mAbs to T-cells in a sample obtained from a healthy individual, a significant difference between the above two extents of binding indicating that said tested individual has a high probability of having a malignant disease.

5 22. An assay according to Claim 21, wherein before step (b) the peripheral blood mononuclear cells (PBMC) are separated, said separated PBMC then being contacted in step (c) with said at least one mAbs of Claims 1-8.

23. An assay according to Claim 21 or 22, wherein said body fluid is blood.

24. An assay for identifying a tested individual with a high probability having a
10 specific malignant disease comprising:

(a) obtaining a body fluid sample from said individual;

(b) contacting said sample with the mAbs of any of Claims 1-8;

(c) determining the extent of binding of said mAbs to T-cells within said sample; and

15 (d) comparing the extent of (c) to the extent of binding of the mAbs to T-cells obtained from a healthy individual, the existence of a significant difference the extents of binding indicating with a high probability that the tested individual has a malignant disease, wherein whether the extent of binding to T-cells of said individual is above or below the level of the binding of the mAbs in T-cells of
20 healthy individuals indicates a specific type of malignant disease which the tested individual has with a high probability

25. An assay according to Claim 24, wherein prior to step (b), PBMCs are separated from said sample, said PBMCs then being contacted in step (c) with said mAbs.

25 26. An assay according to Claims 24 or 25, wherein the extent of binding of the mAbs to T-cells obtained from said tested individual is higher than the extent of binding of the same mAbs to T-cells of healthy individuals.

27. An assay according to Claim 26, wherein said specific malignant disease is prostate carcinoma.

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28. An assay according to Claims 24 or 25, wherein the extent of binding of the mAbs to T-cells obtained from said tested individual is lower than the extent of binding of the same mAbs to T-cells of healthy individuals.
29. An assay according to Claim 28, wherein said specific malignant disease is
5 breast carcinoma.
30. A method according to Claim 28, wherein said specific malignant disease is colon carcinoma.
31. A pharmaceutical composition comprising one or more of the mAbs of Claims 1-8, together with a pharmaceutically acceptable carrier.
- 10 32. A kit comprising one or more of the mAbs of Claims 1-8, together with a conjugate of a specific binding partner for said mAbs, a label capable of producing a detectable signal and directions for its use.
33. A pharmaceutical composition comprising, as an active ingredient, one or more of the mAbs of any of Claims 1-8 together with a pharmaceutically
15 acceptable carrier.
34. A pharmaceutical composition according to Claim 33, for the treatment of cancer.
35. Use of any of the mAbs of Claims 1-8, for the preparation of a pharmaceutical composition for the treatment of a malignant diseases in an
20 individual.
36. A method for the treatment of malignant diseases comprising administering to an individual in need a therapeutically effect amount of one or more mAbs of any of Claims 1-8.
37. A pharmaceutical composition comprising, as an active ingredient, one or
25 more of the peptides of Claims 15-20 together with a pharmaceutically acceptable carrier.
38. A vaccine preparation for immunizing against malignant diseases comprising as an active ingredient, one or more of the peptides of Claims 15-20 together with an immunologically acceptable carrier.

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39. Use of the peptides of any of Claims 15-20 for the preparation of a pharmaceutical preparation for the treatment of malignant diseases.

40. A method for the treatment of a malignant disease comprising administering to an individual in need a therapeutically effective amount of the peptides of any of
5 Claims 15-20.

41. A pharmaceutical composition according to Claim 34, wherein said cancer is a solid tumor.

42. A pharmaceutical composition according to Claim 41, wherein said solid tumor is a prostate carcinoma.

10 43. A pharmaceutical composition according to Claim 41, wherein said solid tumor is a breast carcinoma.

44. A pharmaceutical composition according to Claim 42, wherein said solid tumor is a colon carcinoma.

15 45. A method according to Claim 40, wherein said malignant disease is a solid tumor.

46. A method according to Claim 45, wherein said solid tumor is a prostate carcinoma.

47. A method according to Claim 45, wherein said solid tumor is a breast carcinoma.

20 48. A method according to Claim 45, wherein said solid tumor is a colon carcinoma.

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Fig 1

TACTAGTCGACATGGCTTGGGTGTGGACCTTGCTATTCTGATGGCAGCTGCCCAAAGTATCCAAGCACA
 ATGATCAGCTGTACCGAACCACACACCTGGAACGATAAGGACTACCGTCGACGGGTTTCATAGGTTTCGTGT
 M A W V W T L L F L M A A A Q S I Q A Q
 GATCCAGTTGGTGCAGTCTGGACCTGAGTTGAAGAAGCCTGGAGAGACAGTCAAGATCTCCTGCAAGGCT
 CTAGGTCAACCACGTCAGACCTGGACTCAACTTCTTCGGACCTCTCTGTCAAGTTCTAGAGGACGTTCCGA
 I Q L V Q S G P E L K K P G E T V K I S C K A
 TCTGGATATACTTTCACAAACTATGGAATGAACTGGGTGAAGCAGGCTCCAGGAAGGTTTAAAGTGGA
 AGACCTATATGAAAGTGTGTGATACCTTACTTGACCCACTTCGTCGAGGTCCTTCCCAAATTTACACCT
 S G Y T F T N Y G M N W V K Q A P G K G L K W
 TGGGTGGATAAACACCGACAGTGGAGAGTCAACATATGCTGAAGAGTTCAAGGACGGTTTGCCTTCTC
 ACCCGACCTATTGTGGCTGTCACTCTCAGTTGTATACGACTTCTCAAGTTCCCTGCCAAACGGAAGAG
 M G W I N T D S G E S T Y A E E F K G R F A F S
 TTTGGAAACCTCTGCCAACACTGCCCTATTTCAGATCAACAACCTCAACAATGAGGACACGCCCTACATAT
 AAACCTTTGGAGACGGTTGTGACGGATAAACGCTAGTTGTTGGAGTTGTACTCCTGTGCGGATGTATA
 L E T S A N T A Y L Q I N N L N N E D T A T Y
 TTCTGTGTGAGAGTCGGCTACGATGCTTTGGACTACTGGGTCAAGGAACCTCAGTCACCGTCTCCTCAA
 AAGACACACTCTCAGCCGATGCTACGAAACCTGATGACCCCAAGTTCCCTTGAGTCAAGTGGCAGAGGAGTT
 F C V R V G Y D A L D Y W G Q G T S V T V S S
 CTACAACAACAGCCCCCATCTGTCTATCCCTTCCCGGGTTCCA
 GATGTTGTGTCGGGTAGACAGATAGGGAAGGGCCCCAAGGT

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Fig 2

ACTAGTCGACATGGATTACAGGTGCAGATTATCAGCTTCCCTGCTAATCAGTGCCTCAGTCATAATGTCC
 TGATCAGCTGTACCTAAATGTCCACGCTCTAATAGTCGAAGGACGATTAGTCACGGAGTCAGTATTACAGG
 M D L Q V Q I I S F L L I S A S V I M S
 AGAGGACAAATTGTTCTCACCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAGGTCACCATAA
 TCTCCTGTTTAAACAAGAGTGGGTCAGAGGTCGTTAGTACAGACGTAGAGGTCCTCCCTCTTCCAGTGGTATT
 R G Q I V L T Q S P A I M S A S P G E K V T I
 CCTGCAGTGCCAGGTCAAGTGTAAGTTACATGCACCTGGTTCAGCAGAAAGCCAGGCACCTCTCCCAAAC
 GGACGTCACGGTCCAGTTCACATTCAATGTACGTGACCAAGGTCGTCTTCGGTCCGTGAAGAGGGTTTGA
 T C S A R S S V S Y M H W F Q Q K P G T S P K L
 CTGGATTATAGGACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAAGTGGCAGTGGATCTGGGACC
 GACCTAAATATCCTGTAGGTGGACCCGAAGACCTCAGGGACGAGCGAAGTCACCGTCACCTAGACCCCTGG
 W I Y R T S N L A S G V P A R F S G S G S G T
 TCTTACTGTCTCACAATCAGCCGAATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAAAGGAGTA
 AGAATGACAGAGTGTAGTCGGCTTACCTCCGACTTCTACGACGGTGAATAATGACGGTCGTTTCCTCAT
 S Y C L T I S R M E A E D A A T Y Y C Q Q R S
 GTTCCCACTCACGTTGGCTCGGGGACAAAGTTGGAAATAAAACGGGCTGATGCTGCACCAACTGTATC
 CAAAGGGTGAGTGCAAGCCGAGCCCCCTGTTTCAACCTTTATTTGCCCGACTACGACGTGGTTGACATAG
 S F P L T F G S G T K L E I K
 CATCTTCCCACCATCCAAGATCT
 GTAGAAGGGGTGGTAGGTTCTAGA

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Fig. 3

BAT: QIQLVQSGPELKKPGETVKISCKASGYTFTNYGMN-WVKQAPGKGLKWMG
VMS2:

BAT: WINT-DSGESTYAEFEKGRFAFSLETSANTAYLQINNLLNEDTATYFCVR
VMS2A:NT..P.....S.....K.....A.

BAT: VGYDA-----LDYWGQGTSVTVSS
VMS2: R..YYGSR.....YGAM.....

Residue H 53 (Kabat)

BAT: Asp(D) Germline: Asn(N)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency
Ile	43.33
Pro	25.00
Thr	20.00
Leu	6.67
Ser	1.67
His	1.67
Val	1.67

Residue	% Frequency
Gly	27.60
Asn	25.98
Tyr	13.72
Ser	11.02
Ala	7.33
<u>Asp</u>	<u>4.93</u>
Trp	2.06
Arg	1.84
His	1.63
Glu	1.50
Thr	0.73
Phe	0.47
Lys	0.43
Val	0.26
Leu	0.17
Pro	0.13
Ile	0.09
Gln	0.09
Cys	0.04

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Fig 3 cont.

Residue H 54 (Kabat)
BAT: Ser(S) Germline: Thr(T)

Mouse V _H Misc.		All Mouse V _H	
Residue	% Frequency	Residue	% Frequency
Tyr	30.51	Asn	28.79
Asn	25.42	<u>Ser</u>	<u>26.18</u>
Thr	23.73	Gly	22.72
His	5.08	Asp	13.70
<u>Ser</u>	<u>5.08</u>	Thr	3.94
Lys	3.39	Lys	1.63
Asp	3.39	Tyr	1.34
Leu	1.69	Ile	0.38
Ala	1.69	Arg	0.29
		Ala	0.25
		His	0.25
		Pro	0.17
		Phe	0.13
		Trp	0.08
		Cys	0.08
		Val	0.04
		Leu	0.04

Residue H 57 (Kabat)

BAT: Ser(S) Germline: Pro(P)

Mouse V _H Misc.		All Mouse V _H	
Residue	% Frequency	Residue	% Frequency
Pro	100.00	Thr	77.80
		Ile	11.89
		Pro	2.49
		<u>Ser</u>	<u>2.11</u>
		Lys	1.91
		Asn	1.62
		Ala	0.87
		Leu	0.41
		Val	0.17
		His	0.17
		Arg	0.12
		Gln	0.08
		Met	0.08
		Phe	0.08
		Gly	0.08
		Tyr	0.04
		Cys	0.04
		Asp	0.04

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Fig 3 cont. (2)

Residue H 76 (Kabat)

BAT: Asn(N) Germline: Ser(S)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency	Residue	% Frequency
Ser	58.33	Ser	66.26
<u>Asn</u>	<u>38.33</u>	<u>Asn</u>	<u>29.73</u>
Ile	1.67	Thr	1.42
Tyr	1.67	Arg	0.90
		Lys	0.34
		Asp	0.34
		Gly	0.26
		Ile	0.22
		Tyr	0.17
		Ala	0.13
		Gln	0.09
		His	0.09
		Glu	0.04

Residue H 83 (Kabat)

BAT: Asn(N) Germline: Lys(K)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency	Residue	% Frequency
Arg	51.67	Thr	60.98
Lys	35.00	Arg	18.32
Thr	8.33	Lys	8.69
Gln	1.67	Gln	8.69
Ser	1.67	Asp	1.77
<u>Asn</u>	<u>1.67</u>	Ala	0.91
		Ser	0.35
		<u>Asn</u>	<u>0.13</u>
		Ile	0.09
		His	0.04
		Glu	0.04

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Fig 3 cont. (3)

Residue H 93 (Kabat), Vernier Residue

BAT: Val(V) Germline: Ala(A)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency	Residue	% Frequency
Ala	62.71	Ala	83.26
Met	25.42	Thr	8.03
Thr	3.39	<u>Val</u>	<u>3.74</u>
<u>Val</u>	<u>3.39</u>	Ser	1.36
Lys	1.69	Gly	1.27
Leu	1.69	Met	0.89
Asn	1.69	Asn	0.34
		Pro	0.26
		Lys	0.21
		Trp	0.17
		Asp	0.13
		Ile	0.13
		Leu	0.08
		Tyr	0.04
		Phe	0.04
		His	0.04

Residue H 95 (Kabat)

BAT: Val(V) Germline: Arg(R)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency	Residue	% Frequency
Tyr	25.42	Ser	16.24
Asp	13.56	Tyr	15.18
Trp	11.86	Gly	13.81
Ser	10.17	Asp	13.28
<u>Arg</u>	<u>10.17</u>	<u>Arg</u>	<u>9.00</u>
Gly	6.78	Glu	4.41
Pro	5.08	Asn	4.37
Gln	5.08	Leu	3.53
His	5.08	Pro	2.96
Lys	3.39	His	2.47
Cys	1.69	Ala	2.43
Phe	1.69	Trp	2.38
		Thr	1.94
		Lys	1.68
		Gln	1.50
		<u>Val</u>	<u>1.15</u>
		Ile	1.15
		Phe	1.10
		Cys	1.06
		Met	0.35

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Fig 3 cont. (4)

Residue H 98 (Kabat)

BAT: Asp(D) Germline: Tyr(Y)Mouse V_HMisc.All Mouse V_H

Residue	% Frequency
Tyr	56.00
Gly	10.00
Thr	6.00
Ala	6.00
Asn	4.00
Ser	4.00
<u>Asp</u>	<u>4.00</u>
Leu	2.00
Arg	2.00
Pro	2.00
Ile	2.00
Lys	2.00

Residue	% Frequency
Tyr	32.11
Gly	24.52
<u>Asp</u>	<u>6.79</u>
Ser	6.16
Arg	4.51
Asn	4.41
Thr	3.61
Leu	3.61
Ala	2.18
Phe	2.07
Val	1.70
Trp	1.65
Pro	1.59
Ile	1.22
Lys	1.06
His	1.01
Glu	0.80
Gln	0.69
Met	0.21

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Fig 3 cont. (5)

Residue H 99 (Kabat)

BAT: Ala(A) Germline: Tyr(Y)

Mouse V _H Misc.		All Mouse V _H	
Residue	% Frequency	Residue	% Frequency
Trp	28.26	Gly	27.95
Gly	15.22	Tyr	16.87
Ser	15.22	Ser	16.76
Tyr	13.04	Arg	6.63
Pro	6.52	Asp	4.20
Met	4.35	Asn	4.03
Arg	4.35	Thr	3.73
Asn	4.35	<u>Ala</u>	<u>3.20</u>
Asp	2.17	Leu	3.02
Phe	2.17	Pro	2.78
His	2.17	Phe	2.61
<u>Ala</u>	<u>2.17</u>	Val	2.25
		Glu	1.24
		His	0.89
		Lys	0.89
		Gln	0.83
		Ile	0.83
		Met	0.53
		Trp	0.53
		Cys	0.18
		Other	0.06

Residue H100K (Kabat)

BAT: Leu(L) Germline: Met(M)

Mouse V _H Misc.		All Mouse V _H	
Residue	% Frequency	Residue	% Frequency
Gln	61.54	Phe	70.91
Thr	25.00	Met	24.40
<u>Ala</u>	<u>13.46</u>	<u>Leu</u>	<u>2.46</u>
		Ile	0.59
		Val	0.53
		Ser	0.41
		Tyr	0.35
		Thr	0.18
		Ala	0.06
		His	0.06
		Gly	0.06

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Fig 4

BAT: QIVLTQSPA IMSASPGEKVTITCSARS-----SVSYMHWFQQKPGTSPKLWIY
 H4:S...S.....Y.Y.....S...P...

BAT: RTSNLASGVPARFSGSGSGTSYCLTISRMEAEDAATYYCQQRSSFP-----PLT
 H4:S....S.....YH.Y.....-F.

BAT: FGSGTKLEI-K
 H4:

Residue L 22 (Kabat)

BAT: Thr(T) Germline: Ser(S)Mouse V_xMisc.All Mouse V_x

Residue % Frequency

Residue % Frequency

Thr 98.09
Ser 1.27
 Ile 0.64

Ser 50.26
Thr 46.44
Asn 1.16
 Arg 0.71
 Phe 0.32
 Pro 0.26
 Ala 0.26
 Tyr 0.19
 Ile 0.19
 Cys 0.13
 Other 0.06

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Fig 4 cont.

Residue L 26 (Kabat)

BAT: Arg(R) Germline: Ser(S)Mouse V_xVI

Residue	% Frequency
Ser	96.79
<u>Arg</u>	<u>1.92</u>
Ile	<u>0.64</u>
Thr	0.64

All Mouse V_x

Residue	% Frequency
Ser	96.09
Asn	1.41
<u>Thr</u>	<u>1.28</u>
<u>Arg</u>	<u>0.64</u>
Ile	0.32
Cys	0.06
Met	0.06
Leu	0.06
Gly	0.06

Residue L 34 (Kabat)

BAT: His(H) Germline: Tyr(Y)Mouse V_xVI

Residue	% Frequency
Tyr	85.62
Phe	13.73
<u>His</u>	<u>0.65</u>

All Mouse V_x

Residue	% Frequency
<u>His</u>	<u>35.78</u>
Asn	20.49
Ala	18.73
Tyr	7.89
Glu	6.14
Ser	5.20
Gln	2.00
Thr	1.57
Val	0.38
Phe	0.38
Asp	0.38
Gly	0.38
Ile	0.25
Cys	0.13
Arg	0.13
Trp	0.06
Leu	0.06
Lys	0.06

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Fig 4 cont. (2)

Residue L 36 (Kabat), Vernier Residue

BAT: Phe(F) Germline: Tyr(Y)Mouse V_xVIAll Mouse V_x

Residue	% Frequency	Residue	% Frequency
Gln	98.76	Tyr	82.43
His	1.24	<u>Phe</u>	<u>11.13</u>
		Leu	4.44
		His	0.88
		Asn	0.38
		Ser	0.19
		Cys	0.13
		Val	0.13
		Asp	0.06
		Ala	0.06
		Trp	0.06
		Ile	0.06
		Other	0.06

Residue L 42 (Kabat)

BAT: Thr(T) Germline: Ser(S)Mouse V_xVIAll Mouse V_x

Residue	% Frequency	Residue	% Frequency
Pro	100.00	Gln	44.92
		<u>Thr</u>	<u>18.73</u>
		Lys	10.28
		Gly	9.90
		Ser	7.83
		Glu	2.44
		Ala	1.88
		Phe	1.75
		Asn	1.13
		Arg	0.63
		His	0.19
		Trp	0.06
		Val	0.06
		Pro	0.06
		Ile	0.06
		Other	0.06

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Fig 4 cont. (3)

Val	1.27	Arg	19.36
		Pro	4.53
		Thr	2.94
		Ala	1.23
		Val	0.92
		Ile	0.55
		Phe	0.43
		Gly	0.37
		Ser	0.25
		His	0.12
		Gln	0.06
		Asp	0.06
		Met	0.06
		Lys	0.06

Residue L 72 (Kabat)

BAT: Cys (C) Germline: Ser(S)

Mouse V _x VI		All Mouse V _x	
Residue	% Frequency	Residue	% Frequency
Ser	96.89	Ser	50.52
Thr	3.11	Thr	47.44
		Val	0.56
		Ala	0.37
		Pro	0.31
		Ile	0.31
		Phe	0.19
		Arg	0.12
		Tyr	0.12
		Lys	0.06

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Fig 4 cont. (4)

Residue L 77 (Kabat)

BAT: Arg(R) Germline: Ser(S)Mouse V_xVI

Residue	% Frequency
Ser	83.33
<u>Arg</u>	<u>10.36</u>
Thr	3.85
Pro	1.28
Ile	1.28

All Mouse V_x

Residue	% Frequency
Ser	48.12
<u>Arg</u>	<u>23.28</u>
Asn	13.14
Pro	10.51
Thr	3.69
Gly	0.50
Cys	0.19
Ala	0.13
Asp	0.13
Ile	0.13
Val	0.06
Tyr	0.06
Glu	0.06

Residue L 91 (Kabat)

BAT: Arg(R) Germline: Tyr(Y)Mouse V_xVI

Residue	% Frequency
Trp	80.63
<u>Arg</u>	<u>8.75</u>
Gly	<u>6.25</u>
Phe	1.88
Tyr	1.25
Asp	0.62
Ser	0.62

All Mouse V_x

Residue	% Frequency
Trp	20.41
Gly	19.33
Ser	18.76
<u>Tyr</u>	<u>15.40</u>
His	8.49
Asp	4.37
Phe	3.36
<u>Arg</u>	<u>2.47</u>
Asn	1.90
Leu	1.52
Thr	0.82
Val	0.76
Cys	0.76
Ala	0.63
Ile	0.44
Gln	0.25
Met	0.19
Other	0.13

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Fig. 4 cont. (5)

Residue L 92 (Kabat)

BAT: Ser(S) Germline: His(H)Mouse V_XVIAll Mouse V_X

Residue	% Frequency	Residue	% Frequency
<u>Ser</u>	74.52	<u>Ser</u>	33.10
Asn	12.10	Asn	14.19
Thr	8.92	Thr	12.72
Arg	1.27	Tyr	12.46
Tyr	1.27	His	5.24
Gly	0.64	Asp	4.28
His	0.64	Leu	4.22
Asp	0.64	Arg	3.26
		Trp	2.81
		Lys	1.79
		Ala	1.66
		Gly	1.28
		Val	1.09
		Gln	0.83
		Ile	0.58
		Cys	0.26
		Phe	0.19
		Met	0.06

Residue L 94 (Kabat)

BAT: Phe(F) Germline: Tyr(Y)Mouse V_XVIAll Mouse V_X

Residue	% Frequency	Residue	% Frequency
Asn	66.67	Tyr	19.54
Tyr	15.72	Asn	16.83
Pro	11.32	Val	14.89
Ser	3.77	Leu	13.35
Ile	0.63	Ser	6.90
Leu	0.63	<u>Phe</u>	6.45
Lys	0.63	Thr	6.25
His	0.63	Asp	4.96
		Ile	4.00
		Trp	2.39
		Ala	0.97
		Pro	0.84
		His	0.58
		Gln	0.52
		Met	0.45
		Lys	0.39
		Gly	0.19
		Cys	0.19
		Arg	0.19
		Glu	0.13

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Fig 4 cont. (6)

Residue L 95F (Kabat)

BAT: Pro(P) Germline: Gap(-)

Mouse V _x VI		All Mouse V _x	
Residue	% Frequency	Residue	% Frequency
Arg	100.00	Thr	98.30
		<u>Pro</u>	<u>0.35</u>
		Ser	0.28
		Met	0.21
		Val	0.21
		Phe	0.21
		Leu	0.14
		Ala	0.07
		Arg	0.07
		Lys	0.07
		Other	0.07

Residue L 96 (Kabat)

BAT: Leu(L) Germline: Phe(F)

Mouse V _x VI		All Mouse V _x	
Residue	% Frequency	Residue	% Frequency
<u>Leu</u>	<u>57.14</u>	Phe	99.37
Ile	17.01	<u>Leu</u>	<u>0.28</u>
Tyr	7.48	Gly	0.21
Trp	6.80	Arg	0.07
Phe	5.44	Val	0.07
Pro	2.72		
Arg	2.04		
His	0.68		
Val	0.68		

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Fig 5

AAAGCTTGCCGCCACCATGGATTACAGGTGCAGATTATCAGCTTCCTGCTAATCAGTGCC
TTTCGAACGGCGGTGGTACCTAAATGTCCACGCTAATAGTCGAAGGACGATTAGTCACGG
M D L Q . V Q I I S F L L I S A

TCAGTCATAATGTCAGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCA
AGTCAGTATTACAGGTCCTCGTTTAAACAAGAGTGGGTCAGAGTCGTTAGTACAGACGT
S V I M S R G Q I V L T Q S P A I M S A

TCTCCAGGGGAGAGGTCAACCTGCAGTGCCAGGTCAAGTGAAGTTACATGCAC
AGAGTCCCCCTCTCCAGTGGTATTGGACGTACGGTCCAGTTCACATTCAATGTACGTG
S P G E K V T I T C S A R S S V S Y M H

W F Q Q K P G T S P K L W I Y R T S N L
ACCAAGGTCGCTTCGGTCCGTGAAGAGGGTTTGAGACCTAAATATCCTGTAGGTTGGAC
TGGTCCAGCAGAAGCCAGGCACTTCTCCCAAACCTCTGGATTATAGGACATCCAACCTG

GCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGATCTGGACCTCTTACTGCTCACAA
CGAAGACCTCAGGGACGAGCGAAGTCACCGTCACCTAGACCTGGAGAAATGACAGAGTGT
A S G V P A R F S G S G S G T S Y C L T

ATCAGCCGAATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAAGGAGTAGTTTC
TAGTCGGCTTACCTCCGACTTCTACGACGGTGAATAATGACGGTCGTTTCCTCATCAAAG
I S R M E A E D A A T Y Y C Q Q R S S F

CCACTCACGTTGGGCTCGGGGACAAAGTTGGAATAAAACGTGAGTGGATCC
GGTGAGTGCAAGCCGAGCCCCCTGTTCAACCTTATTTGCACCTCACCTAGG
P L T F G S G T K L E I K

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Fig 6

AAGCTTGCCGCCACCATGGCTTGGGTGGACCTTGCTATTCTGATGGCAGCTGCCCAA
 TTCGAACGGCGGTGCTACCGAACCCACACCTGGAAACGATAAGGACTACCGTCGACGGGTT
 M A W V W T L L F L M A A Q

AGTATCCAAGCACAGATCCAGTTGGTGCAGTCTGGACCTGAGTTGAAGAAGCCTGGAGAG
 TCATAGGTTTCGTCTAGGTCAACCACGTCAGACCTGGACTCAACTTCTTCGGACCTCTC
 S I Q A Q I Q L V Q S G P E L K K P G E

ACAGTCAAGATCTCCTGCAAGGCTTCTGGATATACTTTCACAAACTATGGAATGAACCTGG
 TGTCAAGTTCTAGAGGACGTTCCGAAGACCTATATGAAAGTGTGATACCTTACTTGACC
 T V E I S C K A S G Y T F T N Y G M N W

GTGAAGCAGGCTCCAGGAAAGGTTTAAAGTGGATGGCTGGATAAACACCGACAGTGGA
 CACTTCGTCCGAGGTCCCTTCCCAAATTTCACCTACCCGACCTATTGTGGCTGTACCT
 V K Q A P G K G L K W M G W I N T D S G

GAGTCAACATATGCTGAAGATTCAAGGACGGTTTGCCCTTCTCTTTGGAAACCTCTGCC
 CTCAGTTGTATACGACTTCTCAAGTTCCCTGCCAAACGGAAGAGAAACCTTTGGAGACGG
 E S T Y A E E F K G R F A F S L E T S A

AACACTGCCCTATTGACAGATCAACAACCTCAACAATGAGGACACGGCTACATATTCTGT
 TTGTGACGGATAAACGTCCTAGTTGTTGGAGTTGTACTCCTGTGCCGATGTATAAAGACA
 N T A Y L Q I N N L N N E D T A T Y F C

GTGAGAGTCGGCTACGATGCTTTGGACTACTGGGTCAAGGAACCTCAGTCACCGCTCTCC
 CACTCTCAGCCGATGCTACGAAACCTGATGACCCCAAGTTCCCTTGAGTCAGTGCAGAGG
 V R V G Y D A L D Y W G Q G T S V T V S

TCAGGTGAGTGGATCC
 AGTCCACTCACCTAGG

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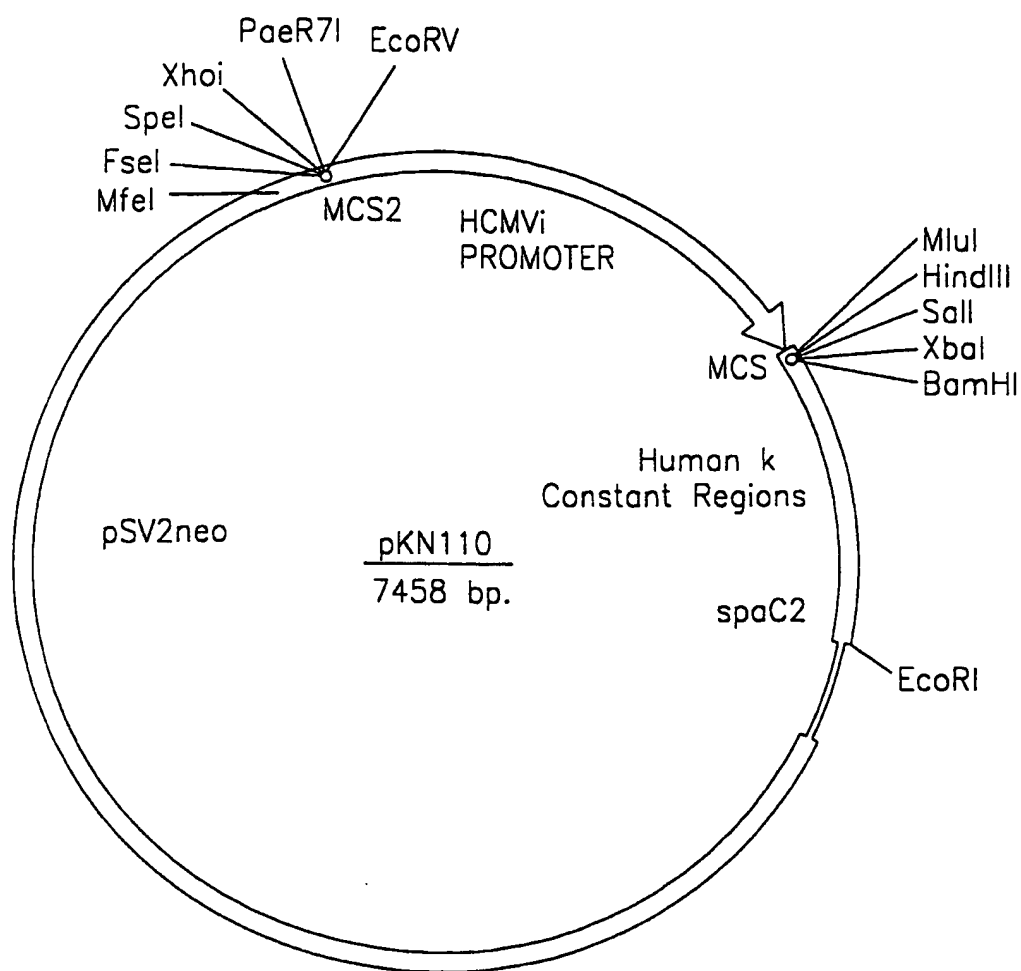


FIG.7

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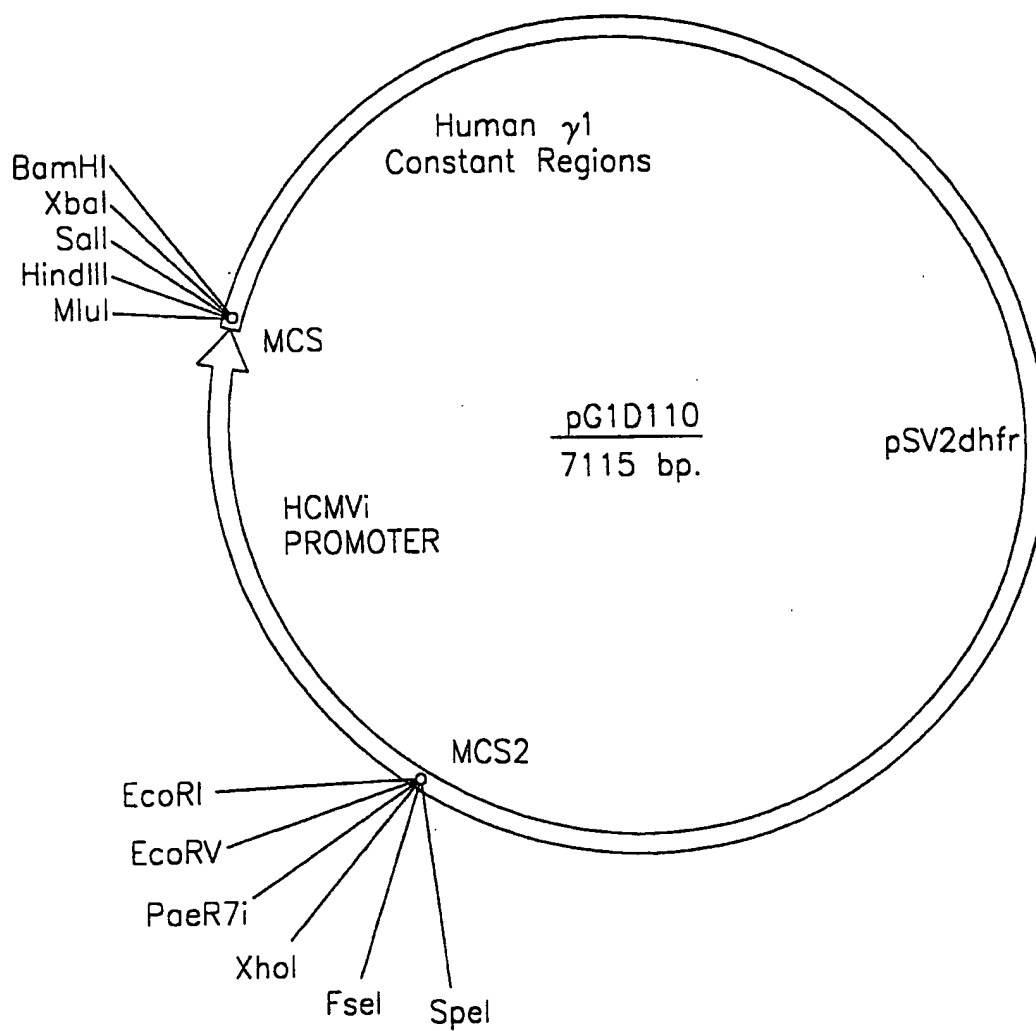


FIG.8

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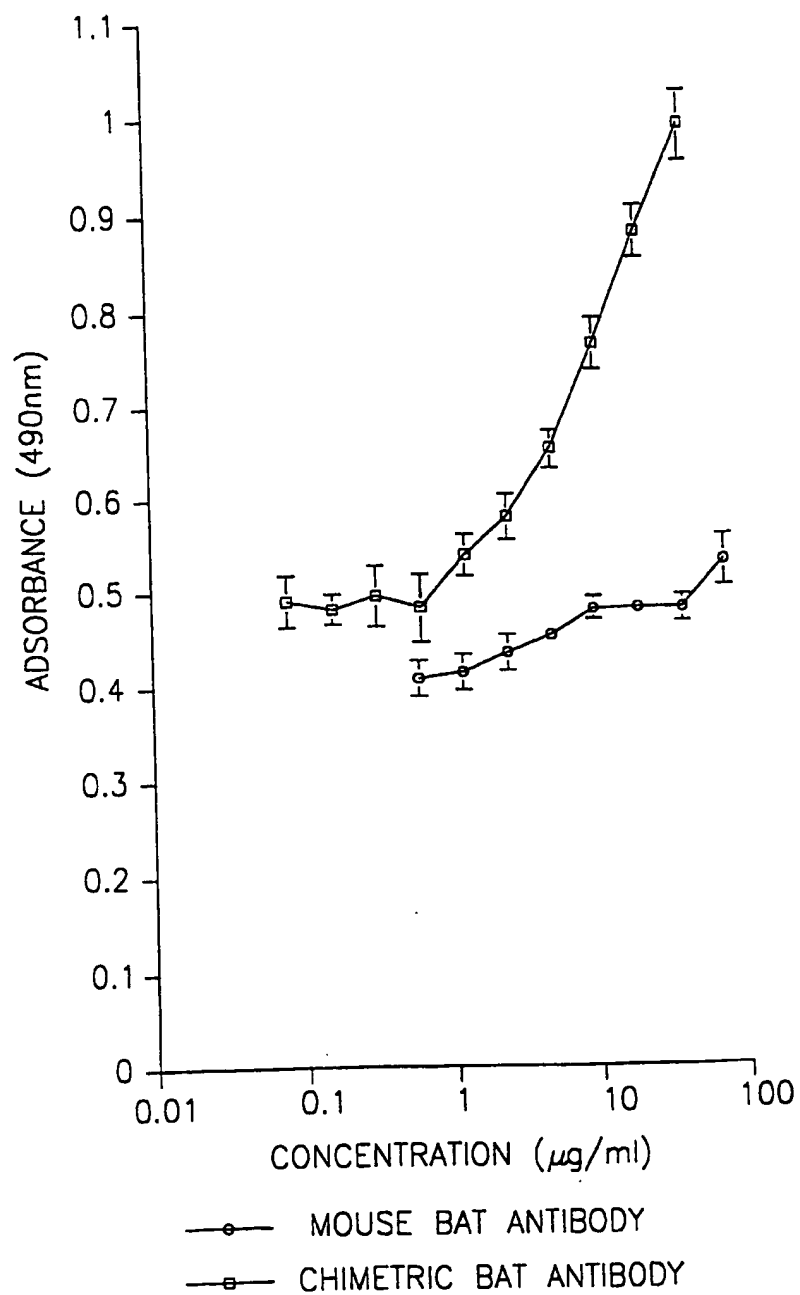


FIG.9

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Peptide 1: TINEEEKC:

FIG.10

Peptide 1: NSGPSMRKKNVSIG

FIG.11

Peptide 1: IPDHQ

FIG.12

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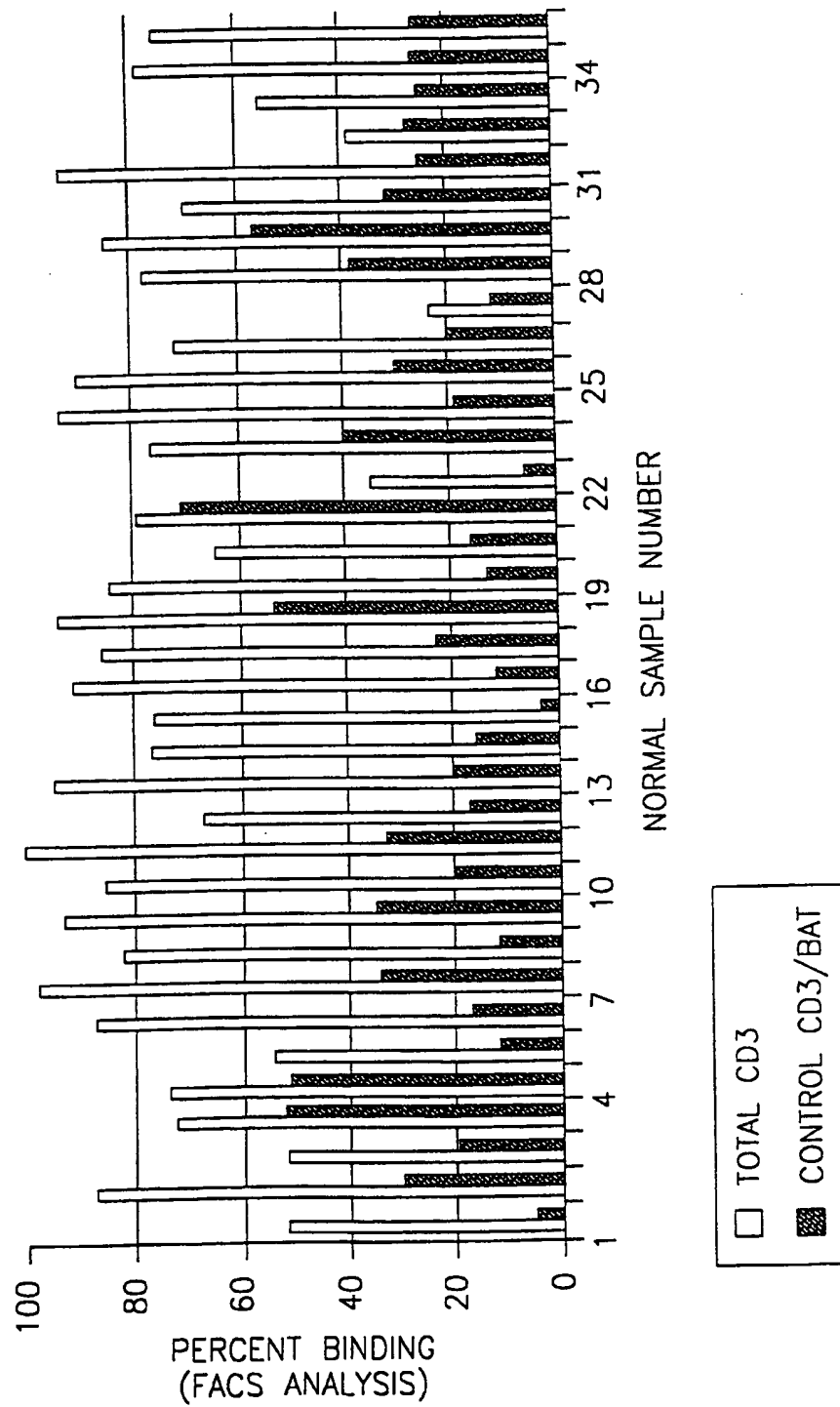
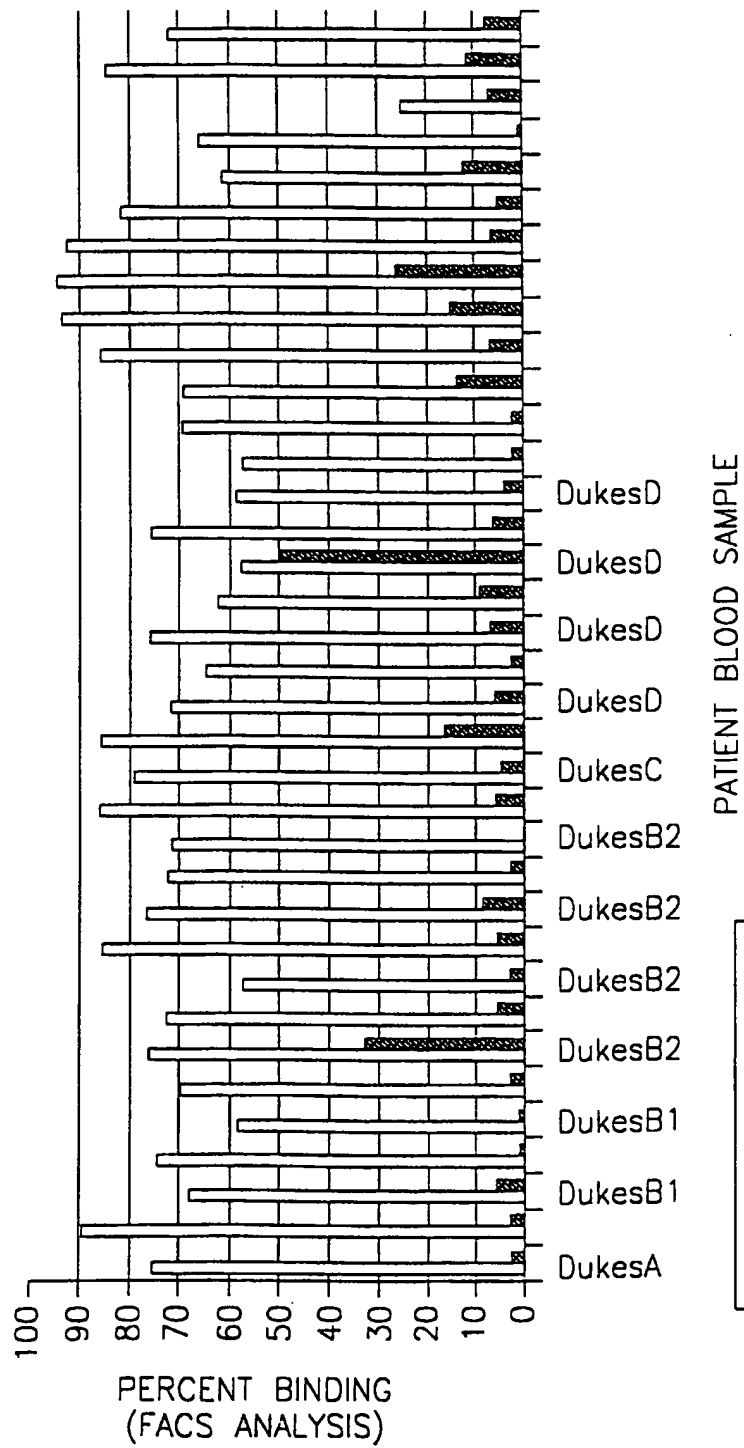


FIG.13

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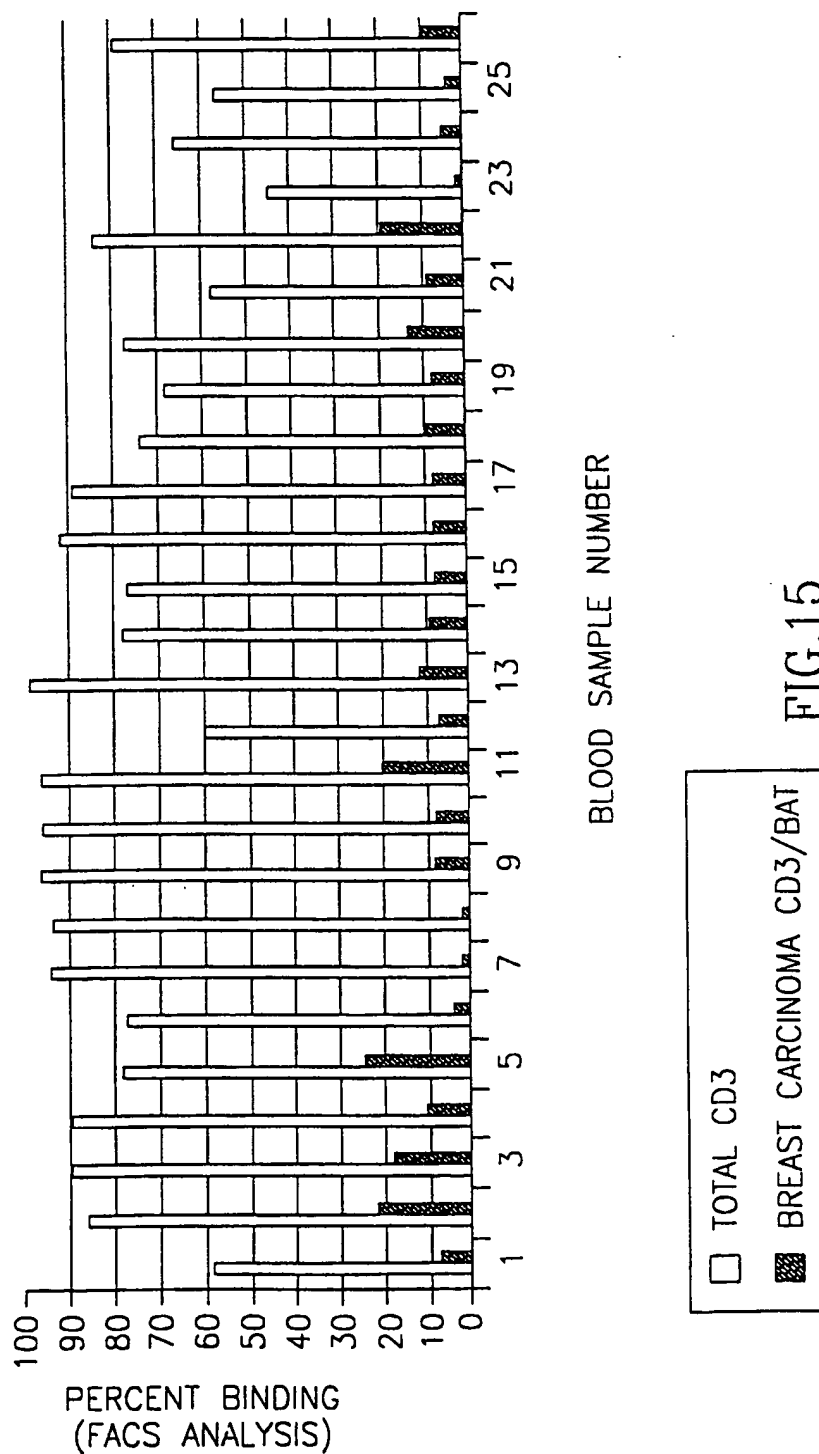


FIG.15

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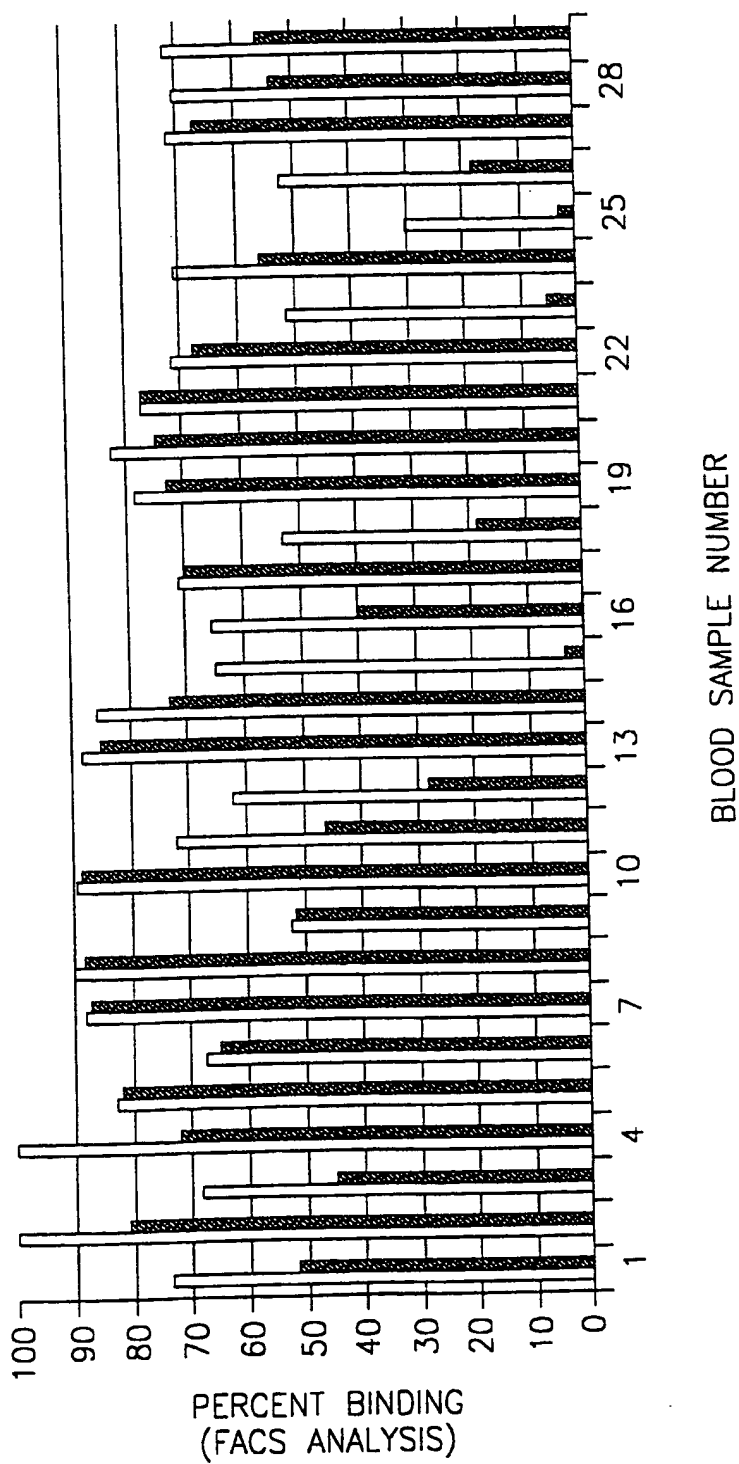
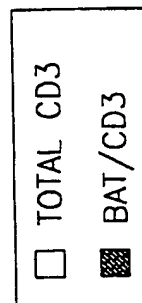


FIG.16



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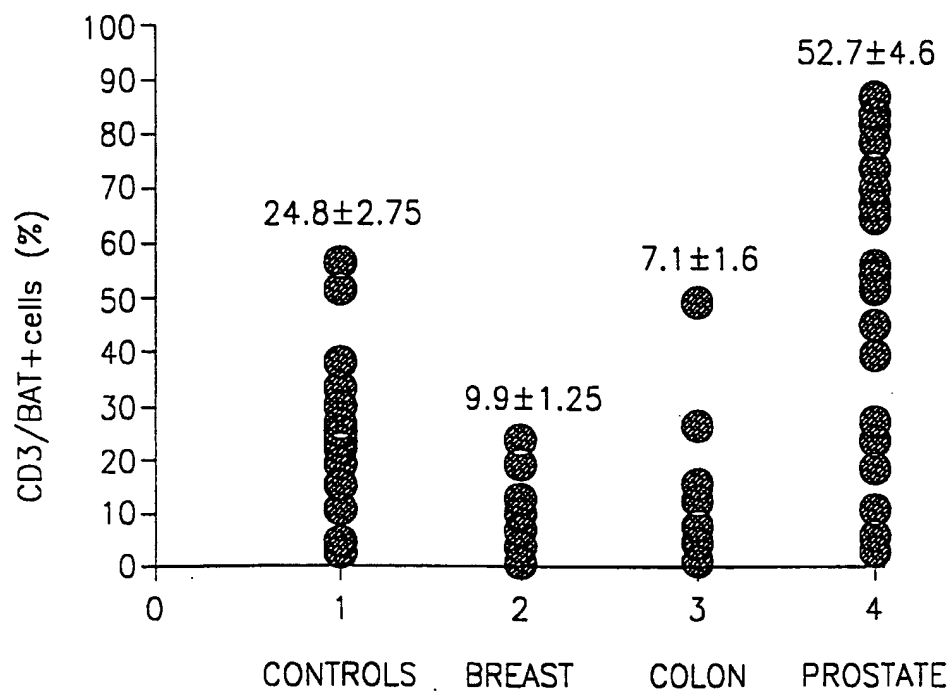


FIG.17

PROSTATE Ca
ENT Ca

PROSTATE Ca

BREAST

DAUDI
MEMBRANES

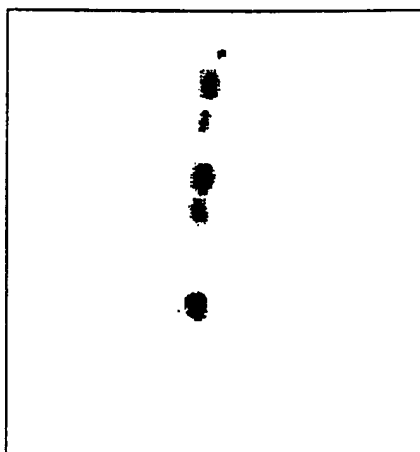


FIG.18

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00518

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/18 C07K16/28 C07K7/04 A61K39/395 A61K38/04
G01N33/574 C12N5/12 C12N5/16 C12N15/13 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARDY B ET AL: "A monoclonal antibody against a human B lymphoblastoid cell line induces tumor regression in mice." CANCER RESEARCH, (1994 NOV 15) 54 (22) 5793-6. , XP000910216 the whole document	9-13
X	HARDY B ET AL: "Activation of human lymphocytes by a monoclonal antibody to B lymphoblastoid cells;molecular mass and distribution o binding protein." CANCER IMMUNOLOGY, IMMUNOTHERAPY, (1995 JUN) 40 (6) 376-82. , XP000910213 the whole document	9-13
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

Date of the actual completion of the international search

27 June 2000

Date of mailing of the international search report

03. 07. 00

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Mennessier, T

INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/IL 99/00518

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 20605 A (MOR RESEARCH APPLIC LTD ;DAVIS JOANNE T (US)) 3 August 1995 (1995-08-03) cited in the application the whole document	9-13
X	--- HARDY B ET AL: "A lymphocyte-activating monoclonal antibody induces regression of human tumors in severe combined immunodeficient mice." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 MAY 27) 94 (11) 5756-60. , XP000910233 the whole document	9-13
X	--- HARDY B ET AL: "Immune stimulatory and anti-tumor properties of anti-CD3 and BAT monoclonal antibodies: a comparative study." HUMAN ANTIBODIES, (1997) 8 (2) 95-8. , XP000915668 the whole document -----	9-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 99/00518

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-8, 9-13 (part) and 14-48
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IL 99/00518

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-8, 9-13 (part) and 14-48

Claims 1-8 and 14-18 were not searched. Claims 9-13 were searched only partly.

A) Claimed not searched

a) Remarks with respect to claims 1-8, 14, 21-36 and 41-44

Claims 1-8 are directed to monoclonal antibodies having a particular amino acid sequence as specified in Figures 1 and 2 or a sequence derived therefrom.

Claim 14 is directed to a hybridoma cell producing any of the monoclonal antibodies of claims 1-8.

Claims 21-30 are directed to assays relying on the use of at least one of the monoclonal antibodies of claims 1-8.

Claims 31, 33 and 34 are directed to pharmaceutical compositions comprising one or more of the monoclonal antibodies of claims 1-8.

Claim 32 is directed to a kit comprising one or more of the monoclonal antibodies of claims 1-8.

Claims 33-34 and 41-44 are directed to pharmaceutical compositions comprising one or more of the monoclonal antibodies of any of claims 1-8.

Claim 35 is directed to the use of any of the monoclonal antibodies of claims 1-8 for the preparation of a pharmaceutical composition.

Claim 36 is directed to a method of treatment comprising administering an amount of one or more monoclonal antibodies of any of claims 1-8.

b) Remarks with respect to claims 15-20, 37-40 and 45-48

Claims 15-20 are directed to peptides having a particular amino acid sequence as specified in Figures 10, 11 and 12 or a sequence derived therefrom.

Claim 37 is directed to a pharmaceutical composition comprising one or more of the peptides of claims 15-20.

Claim 38 is directed to a vaccine preparation comprising one or more of the peptides of claims 15-20.

Claim 39 is directed to the use of the peptides of any of claims 15-20.

Claims 40-45 are directed to methods of treatment comprising administering an amount of the peptides of any of claims 15-20.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IL 99/00518

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

c) Sequence listing filed with the letter of 07.02.2000 in reply to an invitation dated 13.10.1999 under Rule 13ter.1.a) PCT to furnish a sequence listing

Whereas the application contains disclosure of both nucleotide- and amino acid sequences (see Figures, 1, 2, 10, 11 and 12) the sequence listing contains only nucleotide sequences.

Therefore, the sequence listing is incomplete.

d) Rule 13ter.1d) PCT

As the sequence listing is incomplete it has to be considered that it has not been complied with the afore-mentioned invitation, the non-compliance being such that a meaning-full search cannot be carried out with respect to any subject-matter relating to a monoclonal antibody or a peptide or defined with reference to the same.

e) Conclusion

It has not been possible to search claims 1-8 and 14-48.

B) Claims only partly searched

Claim 9 is directed to a nucleic acid sequence encoding the amino acid sequence of any of the monoclonal antibodies of claims 1-8.

Claims 9-13 were searched only insofar as the nucleotide sequence referred to in claim 9 is a sequence as defined in Figures 1 and 2 (which are referred to in claims 1-4).

As the antibodies of claims 5 and 6 are only defined using a vague and indefinite formulation, as reflected by the phrase "at least 70% identity of the amino acid sequence of Fig1/Fig2", which means that each of the said claims encompasses an infinity of different sequences that as such practically is not searchable, it has not been possible to search claims 9-13, insofar as the claimed nucleotide sequence of claim 9 is the nucleotide sequence of either an antibody as defined in claims 5-6 or a chimeric human-mouse antibody derived therefrom as defined in claim 8.

As the antibody referred to in claim 7 is not defined with reference to the sequences of either of Figures 1 and 2, it has not been possible to search claims 9-13, insofar as the claimed nucleotide sequence of claim 9 is the nucleotide sequence of either an antibody as defined in claim 7 or a chimeric human-mouse antibody derived therefrom as defined in claim 8.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 99/00518

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9520605 A	03-08-1995	IL 108501 A	30-10-1998
		AT 171461 T	15-10-1998
		AU 693526 B	02-07-1998
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		SI 742795 T	28-02-1999
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